Toxigenic phytoplankton and concomitant toxicity in the mussel *Choromytilus meridionalis* off the west coast of South Africa in the autumn of 2007

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Preface

The work described in this dissertation was conducted at the Research Aquarium at Sea Point (Marine and Coastal Management), under the co-supervision of Dr. Grant Pitcher (Marine and Coastal Management) and Prof. John Bolton (University of Cape Town) from January 2007 - April 2010. Further analyses were conducted by Dr. Bernd Krock and Dr. Allan Cembella at the Alfred Wegener Institute in Germany and by André Munian at the Council for Scientific and Industrial Research in Cape Town, and are attributed to the relevant contributors in the course of the dissertation.

The content of this dissertation is my own work unless otherwise stated, and has not been submitted in any form for any degree or diploma other than for the degree of Master of Science, in the Department of Botany at the University of Cape Town in 2010. Where reference is made to other people's work it is duly acknowledged.

Abstract

Harmful algal blooms are prevalent off the west coast of South Africa in the summer and autumn months, and have a detrimental impact on human activities along the coast. Some harmful algal blooms are dominated by phytoplankton which produce toxins that can cause shellfish poisoning syndromes. The relationship between the abundance and toxicity of toxigenic phytoplankton, and the concentrations and composition of toxins in the filter-feeding mussel *Choromytilus meridionalis*, was investigated.

The study site was located at a mooring off Lambert's Bay, which was monitored for a period of 22 days (20 March - 10 April 2007). Cell-toxin quotas and toxin profiles for toxigenic phytoplankton, and the uptake and depuration of toxins by the mussel were quantified. Individual toxins were quantified using liquid chromatography linked to tandem mass spectrometry. For the mussel samples toxins were also quantified using a receptor binding assay, an enzyme-linked immunosorbent assay and a mouse bioassay.

Two upwelling events occurred during the survey, during which surface water temperature dropped to a minimum of 11ºC. The first upwelling event was followed by a brief period of relaxation, while the second upwelling event was followed by a longer period of relaxation which lasted until the end of the survey with surface water temperatures finally exceeding 18ºC. Chlorophyll-*a* concentrations reached subsurface maxima of 38 mg.m^{-3}and 63 mg.m^{-3} during periods of relaxation after each upwelling event. These upwelling events resulted in changes in the phytoplankton species assemblage, which included several toxigenic species. The first upwelling event resulted in the decline of a dinoflagellate bloom dominated by *Alexandrium catenella* (Whedon & Kofoid) E. Balach (range 0 - 6.1x10^5 cells.L^{-1}), which was replaced with a diatom bloom immediately after the upwelling event, dominated by *Pseudo-nitzschia* spp. (range 0 - 1.2x10^6 cells.L^{-1}), these were not identified to species level. The diatom-dominated assemblage was later replaced by a mixed assemblage of phytoplankton, which included several *Dinophysis* spp. (range 0 - 8.3x10^4 cells.L^{-1}): *D. acuminata* Claparède & Lachman, *D. fortii* Pavillard, *D. rotundata* Claparède & Lachman, and *D. hastata* Stein. The second upwelling event reset the successional cycle of the phytoplankton assemblage, once again dominated by diatoms including *Pseudo-nitzschia* spp. This assemblage was later replaced with a mixed assemblage of phytoplankton including toxigenic *Dinophysis* spp.

The cell-toxin quota for *A. catenella* (39.4 pg STX eq.cell^{-1}) indicated a highly toxic population, while the cell toxin quotas for *Dinophysis* spp. (0.07 pg OA.cell^{-1}), and *Pseudo-nitzschia* spp. (0.19 pg DA.cell^{-1}) indicated populations of low toxicity.

The toxin profile for *A. catenella* (10% STX; 60% NEO; 25% C1,2; trace amounts of GTX2,3; dcGTX2,3; B1) was dominated by the highly toxic carbamates saxitoxin (STX) and neosaxitoxin (NEO). The composition of these toxins in the mussel (27% STX; 18% NEO; 50% C1,2; trace amounts of dcSTX; GTX1,2,3; B1) showed significantly lower proportions of NEO, and higher proportions of STX and the less toxic N-sulfocarbamoyls C1,2. The toxin profile for the *Dinophysis* spp. (95% OA; 4% DTX1; trace amounts of PTX2) was dominated by okadaic acid (OA). The composition of these toxins in the mussel (77% OA; 18% DTX1; trace amounts of PTX2;

PTX2sa) had significantly lower proportions of OA and and higher proportions of the dinophysistoxin DTX1. *Pseudo-nitzschia* spp. produced only one toxin, domoic acid (DA), which was not detected in the mussel. Biotransformation of toxins between the toxigenic phytoplankton and the mussel was only evident for the dominant toxins, toxins present in trace amounts showed no significant difference.

Toxins produced by *A. catenella* reached a maximum concentration of $7x10^3$ µg STX eq.$100g^{-1}$ mussel, two orders of magnitude above the regulatory limit of 80 µg STX eq.$100g^{-1}$ mussel for shellfish. The onset of depuration by the mussel of the dominant toxins (STX; NEO; C1,2) coincided with low cell concentrations of *A. catenella*, an increased availability of alternative food sources, and an increase in water temperature. The best fit model for depuration of saxitoxin and its derivatives by the mussel was a single phase negative exponential model, with rates of depuration which were highest for the most toxic carbamate derivatives STX and NEO (k = 0.1 - 0.2), intermediate for the least toxic B1 and C1,2 derivatives (k = 0.06 - 0.09), and not significantly different from 0 for the trace toxins dcSTX, GTX1,2,3,4 and dcGTX2,3 ($k < 0.001$). The uptake of the toxins produced by *Dinophysis* spp. (OA; DTX1; PTX2) by the mussel continued steadily for most of the survey period, an increase in the rate of uptake towards the end of the survey period coincided with a persistent population of *Dinophysis* spp., a decrease in the availability of alternative food sources and an increase in water temperature. A maximum of 43 µg OA eq.$100g^{-1}$mussel was recorded, more than double the regulatory limit of 16 µg OA eq.$100g^{-1}$mussel.

Several different methods were used to determine toxin concentrations in the mussel, all of which followed similar trends. Most the methods used to detect the toxins produced by *A. catenella* showed good agreement, although the mouse bioassay tended to underestimate the results. There was particularly good agreement in the methods used to detect the toxins produced by *Dinophysis* spp.

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List of Abbreviations

AOAC Association of Official Analytical Chemists

ASP Amnesic shellfish poisoning

AWI Alfred Wegener Institute

BSA Bovine serum albumin

CTD An instrument that measure Conductivity, Temperature and Depth

DA Domoic acid

DSP Diarrhetic shellfish poisoning

DTX Dinophysistoxin

ELISA Enzyme-linked immunosorbent assay

FDA Organisation for Food and Drug Administration

GTX Gonyautoxins

HAB Harmful algal bloom

HILIC-MS/MS Hydrophilic liquid chromatography linked with tandem mass spectrometry

LC-FD Liquid chromatography linked with fluorescence detection

LC-MS/MS Liquid chromatography linked with tandem mass spectrometry

MBA Mouse bioassay

MOPS 3-(N-morpholino)propanesulfonic acid

NEO Neosaxitoxin

NIO bottle Plastic reversing water bottle first designed at the National Institute of Oceanography

OA Okadaic acid

PMSF Phenylmethylsulfonyl fluoride

PSP Paralytic shellfish poisoning

PTX Pectenotoxins

QC Quality control

RBA Receptor binding assay

RBMP Rat brain membrane preparation

SPE cartridge Solid phase extraction cartridge

STX Saxitoxin

Chapter 1

Introduction

1.1 Background

1.1.1 Upwelling and harmful algal blooms in the southern Benguela

Upwelling in the Benguela current off the west coast of Southern Africa occurs when equatorward winds result in the Ekman transport of surface waters offshore, leading to the transport of deeper nutrient-rich water to the surface. In newly upwelled water the availability of both nutrients and light favours the development of phytoplankton blooms. During relaxation periods the equatorward winds are reduced or reversed, resulting in the poleward and shoreward movement of surface waters (Nelson & Hutchings, 1983).

The cycling of upwelling and relaxation events is not only associated with phytoplankton biomass but also corresponds to changes in the phytoplankton community (Margalef, 1978). Newly upwelled water is turbulent, nutrient-rich and cool. These conditions favour fast growing non-motile phytoplankton such as the diatoms that require mixing to remain within the euphotic zone. As the upwelled water ages and warms due to solar heating it becomes more stratified. These conditions favour motile phytoplankton such as the dinoflagellates, which are able to source nutrients from depth in the absence of mixing. The dinoflagellates may ultimately be replaced by small flagellates as the water becomes highly stratified and nutrients are depleted. This succession can be reset at any time by a new upwelling event.

In some cases developing blooms can be associated with marine faunal mortalities or human illness, in which case they are referred to as harmful algal blooms (HABs). These blooms can be harmful in several ways: either directly by producing toxins that result in mass mortalities of marine fauna (Horstman *et al.*, 1991) and human illness (Grindley & Sapeika, 1969; Horstman *et al.*, 1991; Pitcher *et al.*, 1993, Pitcher & Matthews, 1996) or through physical damage usually to the gills of fish (Grindley & Nel, 1968; Kent *et al.*, 1995); or indirectly through the depletion of oxygen (anoxia) following bloom decay (Grindley & Taylor, 1964; Matthews & Pitcher, 1996). HABs in the Benguela are usually attributed to dinoflagellates, and their harmful impacts are usually associated either with their high biomass, or the production of toxins (Pitcher & Calder, 2000).

The southern Benguela, which extends from Cape Agulhas to Lüderitz, is distinct from the northern Benguela, which extends from Lüderitz to Cape Frio. It is more seasonal, with the equatorward winds that favour upwelling most intense in the spring and summer (Nelson & Hutchings, 1983). Phytoplankton blooms are most prolific during summer and autumn, when the frequency and duration of wind reversals increases and coastal phytoplankton biomass is highest (Pitcher *et al.*, 1992).

The southern Benguela shows spatial as well as seasonal differences in upwelling, owing to variable coastline features, topography and bathymetry (Nelson & Hutchings, 1983; Pitcher & Nelson, 2006). Capes and promontories create embayments downstream where retention time of water is increased. The greater St Helena Bay region downstream of Cape Columbine demonstrates this (Fig. 1.1). Wind-forced upwelling is more intense near the Cape, and together with the alongshore Benguela current forms an equatorward coastal jet that is separated from the coast by the Cape, which effectively separates the nearshore waters to the north of the Cape from the offshore waters. This encourages retention of water within the embayment despite the offshore flow associated with upwelling. The separation of nearshore and offshore coastal waters is illustrated by the clear difference between phytoplankton communities in the nearshore and offshore regions (Pitcher *et al.*, 1992). Additional to the intensified upwelling at the Cape a narrow band of upwelling may extend to the north of Dwarskersbos (Taunton-Clarke, 1985), under upwelling conditions inshore phytoplankton transported away from the coast by the movement of surface waters tend to converge at the dynamic boundary created by the equatorward coastal jet.

The offshore as well as alongshore transport of surface water creates a pressure gradient as sea surface levels rise equatorward and offshore in relation to nearshore and poleward waters (Gan & Allen, 2002). With the relaxation or reversal of winds that cause upwelling water travels down the gradient, creating weak poleward currents. This results in cyclonic circulation in the lee of the Cape and influences the transport and accumulation of coastal phytoplankton blooms (Pitcher & Nelson, 2006).

To the north of Cape Columbine the shelf broadens and is characterised by warmer, more stratified water and higher phytoplankton biomass (Pitcher & Weeks, 2006), particularly during the latter part of the upwelling season when the dinoflagellate contribution to the biomass is highest (Pitcher & Calder, 2000). High phytoplankton biomass (blooms) form as a result of the upwelling and are transported offshore and equatorward yet retained within the embayment. These blooms are accumulated inshore and poleward as relaxation periods between upwelling events increase. The more shallow surface mixed layer in the lee of the Cape favours the formation of motile dinoflagellate blooms that are able to source nutrients below the euphotic zone. The retentive nature of the greater St Helena Bay region also promotes the formation and deposition of dinoflagellate cysts. These cysts are brought back to the surface in subsequent years by upwelling and may act as dinoflagellate bloom innocula (Joyce *et al.*, 2005).

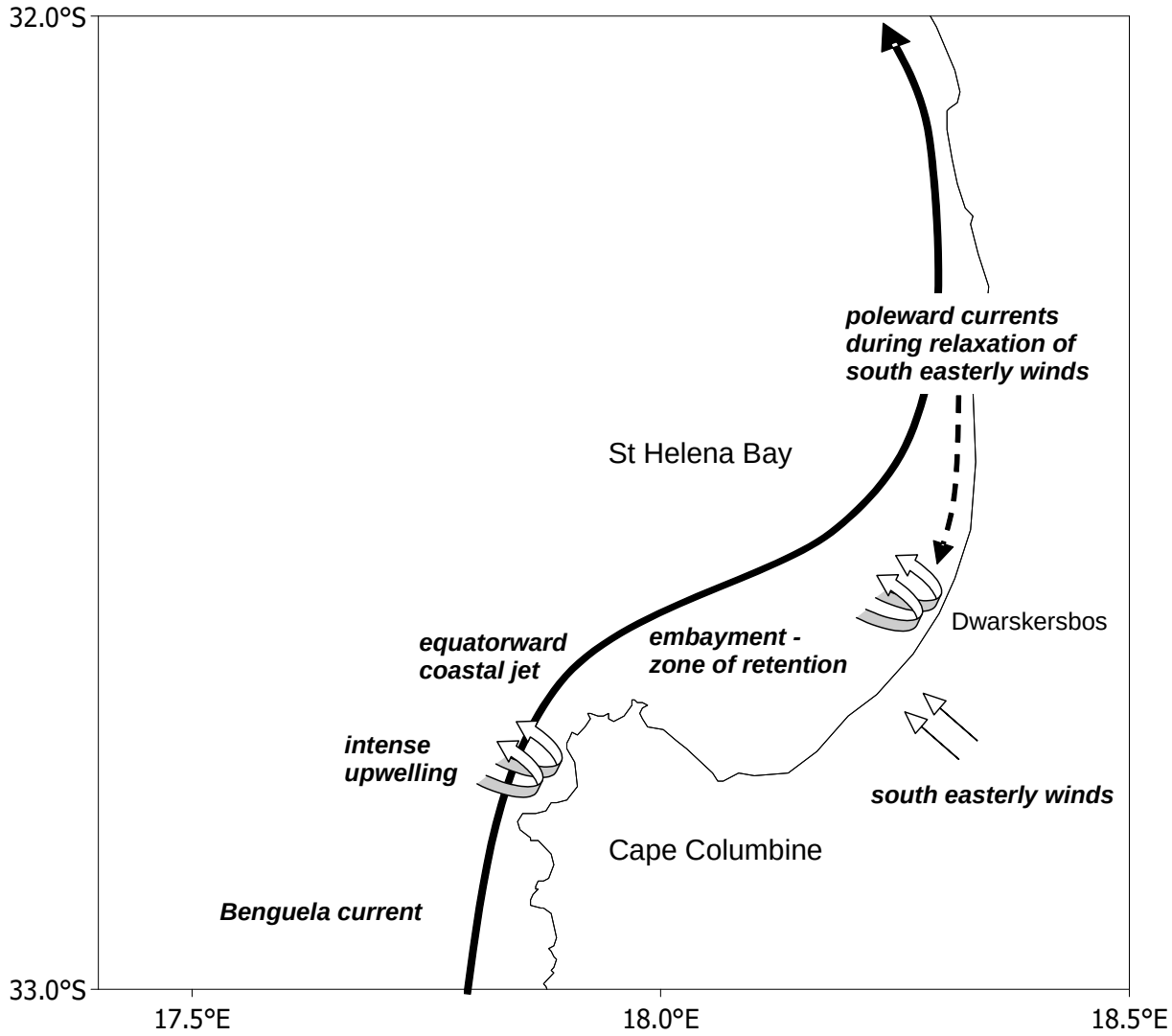


Figure 1.1: Representation of upwelling at Cape Columbine and downstream in the greater St Helena Bay region (Pitcher & Nelson, 2006)

1.1.2 Toxigenic phytoplankton and shellfish poisoning syndromes

Some phytoplankton are toxigenic, producing toxins that are harmful to the organisms that ingest them. The type of toxins produced are specific to the toxigenic phytoplankton and vary in their action, their potency and the duration of their effect. The toxic effect depends on the sensitivity of the organism that ingests the toxin, as well as the type and concentration of the toxin. Organisms such as filter-feeding shellfish that are relatively insensitive to the toxin's effect can accumulate high concentrations of toxins through filtering large volumes of toxigenic phytoplankton. They serve as vectors of toxins and can cause illness and death in the marine fauna and humans that consume the contaminated organisms (Shumway, 1995; Bricelj & Shumway, 1998). Mussels are often used as indicator species for the presence of these toxins in seafood, however they are not the only vectors of these toxins and other shellfish, gastropods and planktivorous fish can also accumulate significantly high concentrations of toxins (Shumway, 1995). There is typically a lag period between the occurrence and disappearance of toxigenic phytoplankton and the uptake and clearance of toxins in shellfish. Rates of uptake and depuration of toxins varies between species of shellfish (Bricelj & Shumway, 1998).

Net production of toxin by toxigenic phytoplankton is referred to as the cell-toxin quota (pg toxin eq.cell^{-1}) and is dependent on internal factors such as metabolic processes in the cell, excretion or loss of toxins by the cell, cell growth and division; as well as external factors such as irradiance, temperature, salinity, turbulence, and substances found in the water (e.g. nutrients and growth promoting substances) (Cembella, 1998). As a consequence the cell-toxin quota is highly variable, even within a single population of toxigenic phytoplankton. The cell-toxin quota acts as a measure of the toxicity of a population of toxigenic phytoplankton (low, moderate or high relative to other recorded values).

Toxigenic phytoplankton that produce more than one chemical derivative of a toxin are considered to have toxin profiles, which are established by the relative proportion of these toxic derivatives. Toxin profiles may vary between different populations or strains of the same species of toxigenic phytoplankton, but within the same population of a single strain the profile is typically conserved, especially at the exponential growth stage (Cembella, 1998). Toxin profiles of toxigenic phytoplankton and the toxin composition found in contaminated shellfish can differ significantly (Oshima *et al.*, 1990) as a result of selective retention and elimination of the individual toxins as well as metabolic processes in the shellfish (Cembella *et al.*, 1993).

Shellfish poisoning syndromes describe various illnesses in humans as a result of consuming toxic seafood (usually shellfish). These syndromes vary in their severity and duration depending on the type and concentration of toxin. In this study the toxigenic phytoplankton that produce the toxins responsible for paralytic (PSP), diarrhetic (DSP) and amnesic shellfish poisoning (ASP) were investigated (Table 1.1).

Table 1.1: Shellfish poisoning syndromes typically found off the west coast of South Africa, the associated toxins and the causative toxigenic phytoplankton found in the southern Benguela, the symptoms of poisoning and the regulatory limits used to ensure that seafood is safe for human consumption (Hallegraeff, 2003)

Shellfish poisoning syndrome	**Toxins**	**Causative toxigenic phytoplankton**	**Symptoms**	**Regulatory limit**
Paralytic shellfish poisoning (PSP)	Saxitoxin and its derivatives (at least 21)	*Alexandrium catenella, A. minutum*	Gastrointestinal – nausea and diarrhoea; Neurotoxic – short term effect (lasting a few hours to a few days) from mild (tingling and numbness) to extreme (coma, death from respiratory paralysis)	80 µg STX eq.$100g^{-1}$ shellfish
Diarrhetic shellfish poisoning (DSP)	Okadaic acid, DTX-1,2	*Dinophysis* spp. (including *D. acuminata*, *D. fortii*, *D. hastata*, and *D. rotundata*)	Gastrointestinal – nausea and diarrhoea; Long term effects may include promoting the formation of tumours	16 µg OA eq.$100g^{-1}$ shellfish
Amnesic shellfish poisoning (ASP)	Domoic acid	*Pseudo-nitzschia* spp.	Gastrointestinal – nausea and diarrhoea; Neurotoxic – from mild (disorientation and short-term memory loss) to severe (seizures)	20 µg DA.g^{-1} shellfish

1.1.2.1 *Alexandrium catenella*, saxitoxins and paralytic shellfish poisoning

Off the west coast of South Africa the toxigenic dinoflagellate *A catenella* is commonly associated with toxins that result in paralytic shellfish poisoning (PSP) (Pitcher & Calder, 2000). *A. catenella* is one of the species in a group of divergent genera of photoautotrophic, planktonic, marine dinoflagellates that are known to produce PSP toxins (Cembella, 1998). *A. catenella* is a morphotype of the "tamarensis group" ecotype within the *Alexandrium* genus, it typically forms chains of 8 cells or more with individual cells ranging from 20-50µm in width (Cembella, 1998; Bricelj & Shumway, 1998).

The cell-toxin quota of *A. catenella* (pg STX eq.cell^{-1}) is known to vary considerably within a natural population, depending on growth and environmental conditions. The cell-toxin quota also varies between populations of the same species that are geographically separated (White, 1986). Cell-toxin quotas are also known to be higher in natural populations compared to cultured isolates of the same strain (Cembella, 1998).

A. catenella typically produces several PSP toxins (Anderson, 1998), the suite of toxins produced by a population is known as the toxin profile. PSP toxins include saxitoxin (STX) and its natural toxic derivatives, approximately two dozen of which have been identified (Shimizu, 1996). These derivatives have different degrees of toxicity, calculated from their relative intraperitoneal toxicity in mice. The most potent form, STX, is assigned the toxicity factor of "1" (Oshima, 1995). The most potent derivatives are the carbamates: saxitoxin (STX), neosaxitoxin (NEO), and the gonyautoxins (GTX) with toxicity factors ranging from 0.36 - 1 . The least potent derivatives are the N-sulfocarbamoyls, including B and C toxins with toxicity factors ranging from <001 - 0.1. Derivatives of intermediate potency are the decarbamoyl and deoxycarbamoyl toxins, indicated by the prefix dc- and do- respectively, with toxicity factors ranging from 0.5 - 0.75. The toxin profile is relatively conserved compared to the cell-toxin quota (Cembella, 1998).

The PSP toxin composition of shellfish often differs from the toxin profile of the causative toxigenic phytoplankton (Oshima *et al.*, 1990). This is as a result of biotransformation, caused by selective elimination and retention of individual toxins, as well as a variety of metabolic processes including epimerisation, reduction, acidic hydrolysis and enzymatic conversion (Bricelj & Shumway, 1998).

PSP toxins are secondary metabolites, with no direct role in the metabolism of the cell (Cembella, 1998) and act by inhibiting ion transport by the sodium channels needed for the functioning of nerves (Narahashi, 1988) by binding to these channels. This binding is highly specific but also completely reversible, which means that while it is a particularly potent toxin there is no long term negative effect once all the toxin has been excreted. Characteristic symptoms of PSP include paresthesia (a tingling and numbness or "pins and needles" sensation), especially in the mouth, and can cause death due to respiratory paralysis (Gessner & Middaugh, 1995).

1.1.2.2 *Dinophysis* spp., the okadaic acid group and pectenotoxins, and diarrhetic shellfish poisoning

Some *Dinophysis* species are associated with the production of the okadaic acid group, which includes okadaic acid (OA) its dinophysistoxin derivatives (DTX), and the pectenotoxins (PTX) (Trainer *et al.*, in press b). Only okadaic acid and the dinophysistoxins are associated with diarrhetic shellfish poisoning (DSP), although pectenotoxins and yessotoxins are often grouped together with the okadaic acid group they do not cause DSP-type symptoms (Trainer *et al.*, in press b; Miles *et al.*, 2004). Okadaic acid is also produced by dinoflagellates other than those in the *Dinophysis* genus. In 1991 *D. acuminata* was the first *Dinophysis* species to be associated with DSP through contaminated shellfish off the west coast of South Africa (Pitcher *et al.*, 1993).

Cell-toxin quotas for *Dinophysis* species can vary widely between populations of the same species that are geographically separated, this could be attributed to different environmental conditions, genetic differences that affect toxin production, or differences in the prey species (plastid sources) (Hackett *et al.*, 2009).

Several natural toxin derivatives of the parent compound OA have been detected, of which two (DTX1,2) are considered toxic, and assigned toxicity factors derived using their relative intraperitoneal toxicity in mice (Quilliam, 2003a). OA is the most potent form, and is assigned the toxicity factor of "1". DTX1 has an equivalent toxicity to OA and is also assigned a toxicity factor of 1, whereas DTX2 is less toxic and is assigned the toxicity factor 0.6 (EFSA-Q-2006-065A, 2008; Aune *et al.*, 2007).

Fifteen derivatives of the parent compound PTX have been been found to date but none have been reported toxic after ingestion or intraperitoneal injection in mice (Miles *et al.*, 2004) and therefore they are not assigned toxicity factors. Pectenotoxins are currently grouped with the okadaic acid group as they often co-occur, although they do not cause DSP-type symptoms .

DSP toxin composition in the contaminated vector species differs from that of the toxigenic phytoplankton. This biotransformation of toxins can be due to selective retention or several metabolic processes, specifically hydrolysis, oxidation and acylation (Moroño *et al*., 2003). Toxic *Dinophysis* species usually occur in relatively low densities in coastal waters compared to other toxigenic phytoplankton, however fairly low densities of a few thousand cells.L^{-1} have contaminated shellfish with sufficiently high concentrations of toxins to result in DSP (Yasumoto *et al.*, 1985). This could be attributed to long retention times for toxins, or the conversion of non-toxic to toxic compounds by shellfish.

DSP toxins inhibit serine/threonine protein phosphatases, an enzyme essential in cell functioning (Bialojan & Takai, 1988). This alteration in cell functioning disrupts the barrier functioning of intestinal epithelial cells, increasing their permeability (Tripuraneni *et al.*, 1997). DSP toxins also lead to submucosal fluid accumulation in the intestinal wall (Hosokawa *et al.*, 1998), which crosses the compromised epithelial layer and enters the intestinal lumen, causing diarrhoea in animals and humans. Long-term low levels of DSP toxins, even when too low to result in immediate symptoms, are also suspected to be carcinogenic (Hallegraeff, 2003).

1.1.2.3 *Pseudo-nitzschia* spp., domoic acid, and amnesic shellfish poisoning

Several *Pseudo-nitzschia* species are associated with the production of domoic acid (DA), a toxin that results in amnesic shellfish poisoning (ASP). ASP outbreaks are mainly recorded in Canada and North America, but while domoic acid has been detected in other parts of the world it is hardly ever present in concentrations high enough to contaminate shellfish significantly (Hallegraeff, 2003). Although toxigenic *Pseudo-nitzschia* species have been observed off the west coast of South Africa there have been no recorded cases of ASP (Pitcher & Calder, 2000).

DA is thought to be produced by cells as a stress response to nutrient limitation and elevated pH, therefore the cell-toxin quota of some toxigenic *Pseudo-nitzschia* species varies considerably from 0 - 100 pg DA.cell^{-1} (Trainer *et al.*, in press).

DA and the eight isodomoic acids (A-H) detected in the marine environment are structurally related to kainic acid (Clayden *et al.*, 2005). Unlike DA, the isodomoic acids show no toxicity when injected intraperitoneally in mice and therefore are not assigned toxicity factors (Munday *et al.*, 2008). Isodomoic acids are sometimes produced together with domoic acid by toxigenic *Pseudo-nitzschia* species, but usually form a relatively small percentage of the toxin profile.

Shellfish contaminated by ASP toxins can have significantly different toxin composition compared to the causative toxigenic phytoplankton. The proportion of the non-toxic isomers compared to the toxic form (DA) often increases (Costa *et al.*, 2005).

Domoic acid (DA) is an amino acid belonging to a group known as the kainoids, neuroexcitants/excitotoxins that disrupt neurotransmission mechanisms in the brain (Quilliam, 2003b). Kainoid toxicity is thought to be due to their structural similarity to glutamic acid, a mammalian central nervous system neurotransmitter (Clayden *et al.*, 2005). The binding of the kainoid in place of glutamic acid leads to neuronal depolarisation, which leads to an increase in cellular Ca^{2+} ions, neuronal swelling and cell death. The targeted nerve cells are located in the hippocampus which is associated with memory retention, hence ASP results in characteristic irreversible short term memory loss (Bates *et al.*, 1998). Severe cases of ASP can result in a coma, leading to either brain damage or death.

1.1.3 Methods for toxin detection

The toxins that cause shellfish poisoning syndromes can be detected using different methods, from analytical methods that quantify individual toxins, to more rapid screening methods that indicate only the presence or absence of toxins. Chemical analytical methods can be used to describe toxin profiles of toxigenic phytoplankton or the biotransformation of individual toxins by the organisms that ingest toxigenic phytoplankton. Other methods require either the use of live organisms (*in vivo* assays) or preparations from live organisms (*in vitro* assays) specific to the toxin or group of toxins to be measured. Bioassays measure the toxic activity of groups of toxins, using functional similarities to group toxins. Immunoassays measure the toxins indirectly through specific binding

to a substrate, using structural similarities to group toxins. While bioassays and immunoassays cannot identify individual toxins, any quantitative method can be used to determine overall toxin concentration of the phytoplankton or of a contaminated vector species, which is expressed as toxin equivalents. Rapid screening methods indicate the presence of toxins above a certain limit but cannot be used to quantify individual toxins or determine toxin concentrations.

Conservative regulatory limits (Table 1.1) for the concentration of toxin equivalents for each group of toxins have been determined, typically using bioassays that measure toxic activity of each group of toxins in mice (Wekell *et al.*, 2004). Adhering to these should prevent both short and long-term ill-effects from eating contaminated seafood.

1.1.3.1 Chemical analytical methods

Chemical analytical methods quantify individual toxins using their chemical properties, usually separating them using chromatographic methods followed by detecting them using fluorometry or mass spectrometry. Different methods are used for hydrophilic toxins such as the saxitoxin group (Luckas *et al.*, 2003), domoic acid (Quilliam, 2003a) and lipophilic toxins such as the okadaic acid group, dinophysistoxins, yessotoxins, pectenotoxins, spirolides and azaspiracids (Quilliam, 2003b). Individual toxin values are then converted into overall toxicity by summing their toxicities, which is calculated from their concentrations and specific molar toxicities.

1.1.3.2 *In vitro* assays

In vitro assays use either the structure of the toxins or their toxic action to quantify the total toxin concentration of contaminated shellfish (Cembella *et al.*, 2003). The enzyme-linked immunosorbent assay (ELISA) uses the structure of the toxins to quantify the total toxin concentration of contaminated shellfish. The toxin in a sample or standard solution competes with an enzyme-linked toxin for binding sites on antibodies raised against the primary form of the toxin. The enzyme-linked toxin changes the colour of a substrate added later, therefore the higher the toxin concentration in a sample or standard solution the lower the colour change (measured using absorbance). The antibodies have a higher affinity for the primary form of the toxin structures compared to its derivatives and therefore ELISA is most quantitative for less structurally diverse toxins.

The receptor binding assay (RBA) quantifies toxins using their toxic effect. The toxin in a sample or standard solution competes with radioactively labelled toxins for binding sites with the receptors which it targets normally in its toxic action. For example neurotoxins such as saxitoxin competes with radiolabelled toxin, the higher the toxin concentration in the sample or standard solution the less radioactive saxitoxin will be bound. The RBA is a relatively sensitive method that relies on toxic activity rather than structure to determine toxin concentrations and is therefore more appropriate for more structurally diverse toxins.

Microtitre plate methods have been developed for most *in vitro* assays in order to facilitate a more rapid analysis of samples.

1.1.3.3 *In vivo* assays

In vivo assays quantify toxicity using the response of animals to toxins (Fernández e*t al.*, 2003). The *in vivo* mouse bioassay (MBA) is a biological assay that quantifies total toxin concentration using toxic action. The response of mice to intraperitoneal injections of standard concentrations of the toxin compared to samples of unknown concentration is used to estimate the amount of toxin present. The MBA is currently the most commonly used method in the routine monitoring of shellfish for shellfish poisoning toxins as it is a robust method, and easily adapted for use in the field. The MBA is particularly appropriate for toxins that are acutely toxic, resulting in fast death times (such as PSP toxins and to a lesser extent, DSP toxins), rather than longer acting toxins (such as ASP toxins).

1.2 Objectives

The objectives of this study were to (1) describe the succession of toxigenic phytoplankton species in association with the physical structure of the water column, (2) determine the cell-toxin quota and toxin profiles of those toxigenic phytoplankton over time, (3) measure the rate of uptake and clearance of toxins by the mussel *C. meridionalis*, comparing different methods for quantifying these toxins and (4) record any biotransformation of toxins by the mussel *C. meridionalis*.

Chapter 2

Methods

A mooring station located on the west coast off Lambert's Bay (32.04°S; 18.26°E, Fig. 2.1) within the greater St Helena Bay region, was sampled on 23 consecutive days during the autumn of 2007, from the 20 March - 11 April. A Seabird CTD was used to profile the water column, including water temperature. Discrete water samples were collected for chlorophyll-*a* analysis, as well as the identification and enumeration of toxigenic phytoplankton for the establishment of cell-toxin quotas and toxin profiles. Meshed bags containing mussels (*Choromytilus meridionalis*) harvested from Malkopbaai were suspended from the mooring at the beginning of the survey period and harvested daily to assess the uptake and depuration of toxins.

2.1 Water temperature profiles

A Seabird CTD was used to profile water temperature daily at the mooring off Lambert's Bay to a depth of 20 m. The water temperature profile provided a clear indication of the upwelling and downwelling cycles as dictated by the influence of wind stress on the surface boundary layer, and of the mixing-stratification gradient important in determining the phytoplankton assemblage.

2.2 Chlorophyll-*a* concentration

Water samples for chlorophyll-*a* analysis were collected from discrete depths, at 5 m intervals from the surface to 20 m by means of NIO bottle samples. Chlorophyll-*a* concentration provided a measure for total phytoplankton biomass. Chlorophyll-*a* concentrations were determined by fluorometric analysis using the method described in Parsons *et al.* (1984).

Samples of 100 ml were filtered through Whatman® GF/F filters. These filters were extracted for 24 hours in 90% acetone at -20°C, in capped acetone resistant test tubes. The acetone extracted samples were centrifuged, the supernatant was transferred into a cuvette and read using a 10-AU

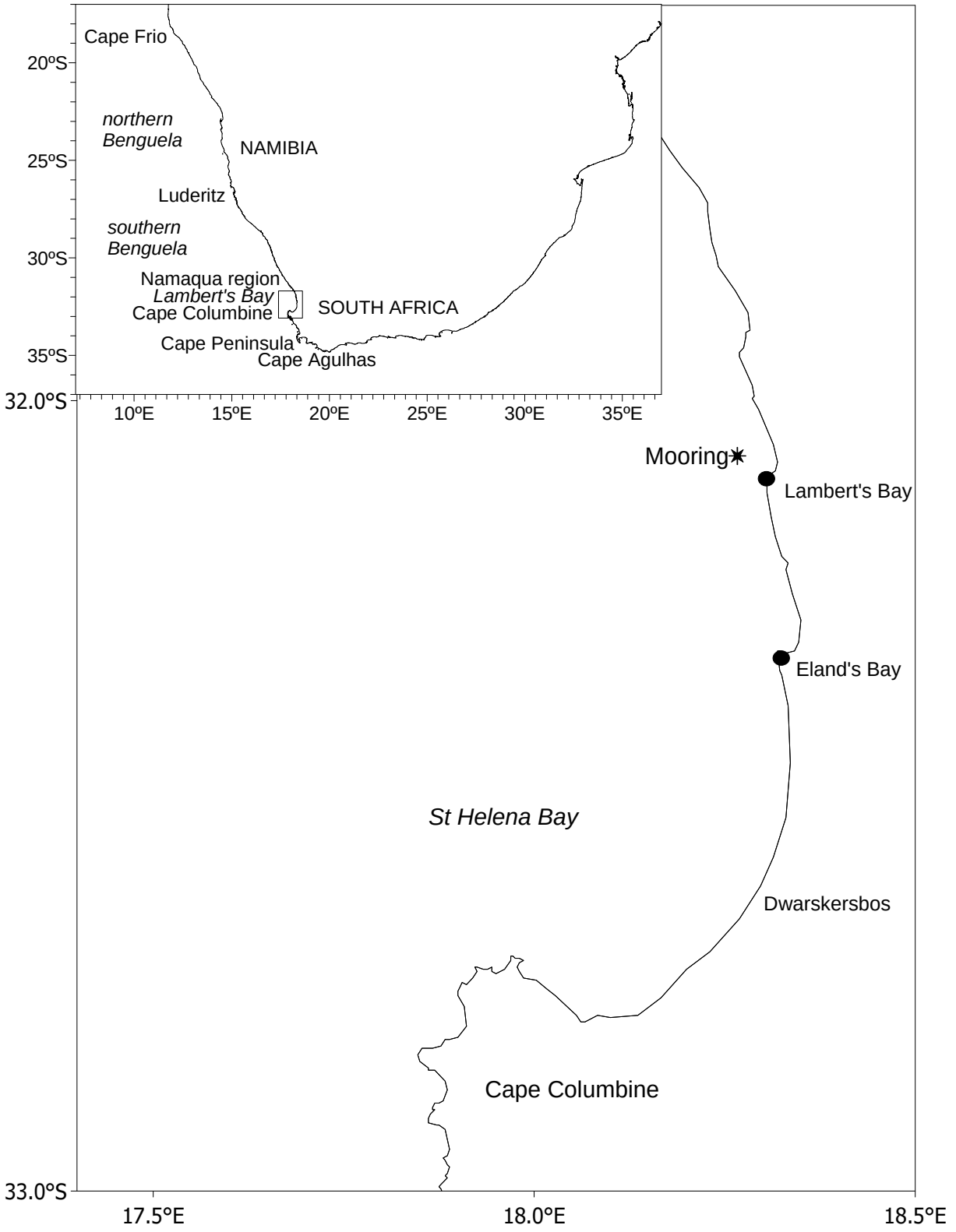


Figure 2.1: Position of mooring (32.04°S, 18.26°E) on the west coast of South Africa off Lambert's Bay.

Turner Designs fluorometer before and after acidification. Samples were acidified by adding 0.3 ml 1 M hydrochloric acid. If required, samples were diluted further with 90% acetone in order to get accurate readings.

The chlorophyll-*a* concentration was calculated using the formula:

$$[\text{chl-}a](\text{mg.m}^{-3}) = \text{K} \times \frac{\text{r}}{\text{r}-1} \times (\text{R}_b - \text{R}_a) \times \frac{\text{V}_{ext}\ (\text{ml})}{\text{V}_{fil}\ (\text{ml})} \times \text{D}$$

Where

[chl-*a*] = chlorophyll-*a* concentration

K = sensitivity co-efficient (1 for Turner 10-AU)

r = max. acid ratio R_b/R_a for chlorophyll-*a* standard

R_b= reading before acidification

R_a= reading after acidification

V_{ext}= extraction volume

V_{fil}= volume sample filtered

D = dilution factor

2.3 Cell concentrations

The water samples used for chlorophyll-*a* determination were also used to identify and enumerate toxigenic phytoplankton, which were identified using light microscopy and grouped according to the types of toxin they produced. The toxigenic phytoplankton observed during this study include *Alexandrium catenella*, *Dinophysis* spp. including *D. acuminata*, *D. fortii*, *D. hastata* and *D. rotundata*, and *Pseudo-nitzschia* spp. Cell concentrations of these toxigenic phytoplankton were estimated for each depth sampled using the Utermöhl method as modified by Hasle (1978).

Samples of 200 ml were fixed in 2.5% buffered formalin (pH 7.5 - 8) from which sub-samples of 2, 5, or 10 ml were settled overnight. Samples with lower cell concentrations required higher volumes to be settled. The toxigenic phytoplankton in the samples were counted under either 200X or 400X total magnification using an inverted light microscope. Several transects of the settling chamber were counted, until at least 30 - 40 cells were recorded. Cell concentrations were calculated using the formula:

$$[\text{cell}](\text{cells.L}^{-1}) = \text{count (cells)} \times \frac{A_{tot}}{A_{trans} \times n_{trans}} \times \frac{1}{V(\text{ml})} \times \frac{1000 \text{ ml}}{1 \text{ L}}$$

Where

[cell] = cell concentration

A_{tot}= total area of the chamber

A_{trans}= area of transect

n_{trans}= no. of transects

V = volume settled

2.4 Toxicity

Both phytoplankton and mussel samples were analysed for toxicity. Samples of 200 ml from 0 m and 5 m were filtered through Whatman® GF/F filters, the filters were placed in cryogenic vials, frozen in liquid nitrogen and stored at -80°C before analysis. Approximately 700 adult mussels of uniform size and of the same species (*Choromytilus meridionalis*) were harvested from the rocks off Malkopbaai, (32.06ºS; 18.18ºE), on 19 March 2007. Malkopbaai was chosen as the site for mussel collection due to its proximity to the mooring, approximating the environmental conditions found at the mooring (particularly the toxic phytoplankton present in the water). This nearby site was also chosen to reduce the effect of a delay between collection and deployment, such as the depuration of toxins already present in the mussels. An initial sample of 30 mussels were kept aside to analyse their toxin content, the remaining mussels were distributed into wide mesh bags with ~30 mussels per bag and suspended from the mooring off Lambert's Bay, at a depth of approximately 2 m. One bag of mussels was harvested daily and stored at -20°C before analysis.

Mussel samples were prepared before extraction according to the standard method described in the Official Methods of Analysis, AOAC (1990). All the flesh was removed from the shell which was opened by cutting the adductor muscles and tissue at the hinge. The mussel flesh was drained by placing it onto a 2 mm aperture Madison Test Sieve without layering for 5 minutes, and homogenised in a Waring commercial blender for a few minutes at room temperature. Sub-samples of 20 g homogenate were decanted into centrifuge tubes and stored at -80ºC before analysis.

Analyses used for quantifying toxin content in the phytoplankton and mussel samples included analytical methods which quantified individual toxins, as well as *in vitro* and *in vivo* assays which quantified total toxin content based on either their structure or their toxic action. The analytical method, liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) or fluorescence detection (LC-FD), detected individual toxins based on their structure, and was used for the analysis of both phytoplankton and mussel samples. *In vitro* assays quantify toxins using the toxin's structure or toxic action. The enzyme-linked immunoassay (ELISA) is based on the structure of the toxins, and was used to quantify PSP and DSP toxins in the mussel samples, while the

receptor binding assay (RBA) is based on the toxic action, and was used to quantify PSP toxins in mussel samples. The *in vivo* mouse bioassay (MBA) quantifies toxins based on their toxic action, and was also used to quantify PSP toxins in mussel samples.

2.4.1 Liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) or fluorescence detection (LC-FD)

Phytoplankton and mussel toxin content was quantified for each of the samples using liquid chromatography linked with tandem mass spectrometry (LC-MS/MS), for PSP toxins fluorescence detection (LC-FD) was also used. These analyses were conducted by Dr. Allan Cembella and Dr. Bernd Krock at the Alfred Wegener Institute (AWI) in Germany, as described by Krock *et al.* (2008; 2009), Fawcett *et al.* (2007) and Pitcher *et al.* (2007).

2.4.1.1 Extraction for PSP toxins

PSP toxins in phytoplankton samples were extracted in 0.1 M acetic acid by sonication as described by Parkhill & Cembella (1999).

PSP toxins were extracted from mussel samples as described in Dell'Aversano *et al.* (2005). Mussel homogenate (5 g) was extracted with 10 ml acetonitrile/water (80:20, v/v) with 0.1% formic acid. The mussel samples were homogenised at 10 000 rpm for 5 minutes and centrifuged at 7 000 rpm for 10 minutes. The supernatant was removed and the pellet was re-extracted twice over with 5 ml of acetonitrile as above. The supernatants were combined and transferred into a solid phase extraction (SPE) cartridge for cleaning. The SPE cartridge had been previously conditioned with 5 ml acetonitrile/water (10:90, v/v) with 0.1% formic acid, and 5 ml acetonitrile water (90:10, v/v) with 0.1% formic acid. 1 ml of the crude extract was loaded into the cartridge and washed with 1 ml acetonitrile water (80:20, v/v) with 0.1% formic acid, and 0.4 ml acetonitrile/water (10:90, v/v) with 0.1% formic acid. PSP toxins were eluted into a 2 ml volumetric tube using acetonitrile/water (10:90, v/v) with 0.1% formic acid.

2.4.1.2 Extraction for ASP and lipophilic toxins (DSP and pectenotoxins)

ASP and lipophilic toxins in phytoplankton samples were extracted as described in the Stobo *et al.* (2005) method for rapid multiple toxin analysis of lipophilic shellfish toxins. Filters were cut into strips, which were transferred into FastPrep Tubes with 0.9 g of lysing matrix D (Thermo Savant, Illkirch, France) and 0.5 ml methanol. The filters were homogenised in a FastPrep Instrument (Thermo Savant, Illkirch, France) for 45 seconds at 6.5 $m.s^{-1}$ (maximum speed). After homogenisation samples were centrifuged at 4°C at 16 000 g for 15 minutes. The supernatant was removed and the pellet re-extracted with 0.5 ml methanol as above. The supernatants were combined and transferred into spin filters with 0.45 µm pore size (Millipore Ultrafree, Eschborn, Germany), centrifuged at 800 g for 30 seconds and transferred into autosampler vials.

Triplicate samples of 2 g mussel homogenate were weighed into 50 ml centrifuge tubes and ASP and lipophilic toxins were extracted with 5 ml methanol/water (80:20, v/v). The mussel samples were homogenised in a Ultraturrax homogeniser (IKA, Staufen, Germany) at maximum speed. After homogenisation mussel samples were centrifuged at 2 000 g for 15 minutes. The supernatant was removed and the pellet re-extracted with 5 ml methanol as above. The supernatants were combined and the toxins were extracted by adding 7 ml *n*-hexane and homogenised in the Ultraturrax homogeniser for 2 minutes. The lipid extract was centrifuged at 2 000 g for 10 minutes and the hexane phase was discarded. The lipid extraction was repeated twice and for final extraction of lipophilic toxins 3.5 ml of double distilled water and 7 ml chloroform was added and the sample was homogenised for 2 minutes. After homogenisation the samples were centrifuged at 2 000 g for 15 minutes and the chloroform layers were collected in 25 ml flasks. The chloroform extraction was repeated and the combined extract collected was dried overnight using 500 mg anhydrous sodium sulfate. The organic phases were collected in separate 25 ml flasks and the residues were washed twice with 1 ml chloroform. The combined extracts were dried via evaporation using a rotary evaporator (Büchi, Konstanz, Germany) and 1 ml methanol was added. The extract/methanol mix was transferred to spin filters with 0.45 µm pore size, centrifuged at 800 g for 30 seconds and transferred to autosampler vials.

2.4.1.3 LC-FD for PSP toxins

PSP toxins saxitoxin and its derivatives were quantified using liquid chromatography linked with fluorescence detection (LC-FD), following post-column oxidation of the toxin derivatives, according to the method described in Diener *et al.* (2006) with minor modifications. Individual PSP toxins were separated using reverse-phase ion-pair chromatography on an Agilent LC1100 liquid chromatograph. A 5 µl volume of extract was injected at a flow rate of 1 ml.min^{-1} into a Phenomenex Luna C18 column (5 µm, 250 mm x 4.6 mm). The individual toxins were separated using two eluents (A and B). Eluent A consisted of 6 mM octanesulphonic acid, 6 mM heptasulphonic acid, 40 mM ammonium phosphate and 0.75% tetrahydrofuran. Eluent B consisted of 13 mM octanesulphonic acid, 50mM phosphoric acid (pH 6.9, adjusted using ammonium hydroxide), 15% acetonitrile and 1.5% tetrahydrofuran. From 0 - 15 min the mobile phase was 100% A, 16 - 35 min 100% B, 36 - 45 min 100% A. The final eluents were degassed for 15 minutes in an ultrasonic bath.

A Pickering PCX 2500 post-column reaction module was used for post-column derivitisation. The derivitisation used a solution of 10 mM periodic acid and 550 mM aqueous ammonia and 0.75 M aqueous nitric acid (to lower pH to 2 - 3) at a flow rate of 0.4 ml.min^{-1}.

After post-column derivitisation, fluorescence detection was used to determine PSP oxidation products. The Agilent G1321A fluorescence detector with dual monochromator was set at fixed wavelengths for excitation (333 nm) and emission (395 nm).

2.4.1.4 HILIC-MS/MS for PSP toxins

Hydrophilic interaction liquid chromatography linked with tandem mass spectrometry (HILIC-MS/MS) described in Quilliam *et al.* (2001) was used to confirm the structures of PSP toxins. The Agilent LC1100 liquid chromatograph was used in conjunction with an ABI-SCIEX API-165 single-quadrupole mass spectrometer with an ion spray source. 10% of the column effluent was directed into the mass spectrometer. The mass spectrometer operated in positive ion mode using both full scan and selected reaction monitoring (SRM). Nitrogen, at a collision energy of 20 eV, was used as the collision gas. A 30 µl volume of extract was injected at a flow rate of 0.2 ml.min^{-1} into a TosoHaas TSK-GEL Amide-80 column (5 µm, 250 mm x 2 mm). The mobile phase was acetonitrile/water (62:28, v/v) with 2 mM ammonium formate and 3.5 mM formic acid.

2.4.1.5 LC-MS/MS for ASP and lipophilic toxins (DSP and pectenotoxins)

ASP and lipophilic toxins (including DSP toxins and pectenotoxins) were quantified using liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) described in Stobo *et al.* (2005) with modifications. The chromatograph was used in conjunction with An ABI-SCIEX API-4000 QTrap (Darmstadt, Germany) triple-quadrupole mass spectrometer with a turbo ion spray source. The mass spectrometer operated in negative ion mode. Extract was injected at a flow rate of 0.3 ml.min^{-1} into a Hypersil BDS C8 column (3 µm, 50 mm x 2 mm). The individual toxins were separated using two eluents (A and B). Eluent A consisted of water and eluent B consisted of 95% acetonitrile/methanol (1:2, v/v) and 5% water. Both eluents contained 2 mM ammonium formate and 50 mM formic acid. From 0 - 6 minutes the mobile phase changed along a linear gradient from 40% B to 100% B, 6 - 15 minutes 100% B, thereafter it returned to 40% B.

2.4.1.6 Analysis of toxicity results

Individual toxin values were converted to total toxin equivalents using conversion factors inferred from specific toxicity values given by Oshima (1995). Total toxin or total toxin equivalents for the toxigenic phytoplankton together with estimated cell density were used to calculate the cell-toxin quota (measured as pg toxin eq.cell^{-1}). Individual toxin values were also used to determine the toxin profiles of the toxigenic phytoplankton and toxin composition for the mussel *Choromytilus meridionalis*.

2.4.2 Enzyme-linked immunosorbent assay (ELISA)

PSP and DSP toxins in the mussels were also quantified using enzyme-linked immunosorbent assays (ELISA), an *in vitro* assay which identifies toxins based on their structure. This method was previously too time consuming for large numbers of field samples, however new microtiter plate methods make it possible for many samples to be analysed simultaneously. In the direct competitive ELISA toxins present in the standard solution (of known toxin concentration) or the sample (of

unknown toxin concentration) and a toxin-enzyme conjugate compete for binding sites with antibodies raised against the toxin. The toxin antibodies are then bound by a second antibody raised against the toxin antibodies, which is immobilised on the plate. Unbound toxins and toxin-enzyme conjugates are washed from the plate and a substrate solution is added. A colour change of the substrate is catalysed by the enzyme in the toxin-enzyme conjugate, which can be measured by its absorbance of light at a specific wavelength. The intensity of the colour is inversely proportional to the amount of toxin present in the standard solution or sample, as higher toxin concentrations would lead to less toxin-enzyme conjugates binding to the antibodies. The unknown toxin concentration in the mussel samples is then determined by comparing their absorbance readings to a standard curve relating concentration of the toxin to absorbance, sample extracts are diluted until the absorbance readings are within the linear part of the standard curve.

Commercially available test kits from Abraxis (Product No. 52255B for PSP toxins and 520021 for DSP toxins) were used to perform the ELISA, and were stored at 4ºC before use. The ELISA for PSP and DSP toxins were conducted based on the methods described by Chu & Fan (1985) and Uda *et al.* (1988) respectively.

2.4.2.1 Extraction of mussel samples

For PSP toxins 10 g mussel homogenate was extracted using 10 ml 0.1 M hydrochloric acid, followed by heating until boiling point for 5 minutes while stirring, and allowed to cool. The pH was adjusted to pH < 4.0, raising the pH with 5 N hydrochloric acid, or lowering it with 0.1 N sodium hydroxide. The sample extract was centrifuged at 3 500 g for 10 minutes and the supernatant was decanted and diluted 1 000X by making 10 µl extract up to 10 ml with Sample Dilution Buffer.

To extract DSP toxins, 6 ml methanol/deionised water (80:20, v/v) was added to 1 g mussel homogenate. The sample extract was centrifuged at 3 000 g for 10 minutes and the supernatant was decanted. The pellet was re-extracted as above using 2 ml of methanol and the supernatants were collected. These supernatants were made up to 10 ml with methanol/deionised water (80:20, v/v), resulting in a 10X dilution of the original extract. The extract was filtered through 0.45 µm nylon syringe filters. A 10 µl volume of filtered extract was made up to 1 ml using Sample Dilution Buffer, a further 100X dilution which results in a final dilution of 1 000X. The diluted sample extract was stored at -20°C before analysis.

If necessary samples were diluted further until readings were within the range of the standard curve: from 0 - 0.4 ng STX.ml^{-1} for PSP toxins, and 0 - 5 ng OA.ml^{-1} for DSP toxins.

2.4.2.2 Direct competitive assay

PSP and DSP toxins were detected using a direct competitive assay. Duplicates of standard solutions and extracted samples were incubated at room temperature for an hour, together with toxin-enzyme conjugates (STX-enzyme conjugate for PSP toxins and OA-enzyme conjugate for DSP

toxins) and antibodies against the toxins. Toxins in the standard solution and the extract samples compete directly for binding sites with an antibody against the toxin, which binds with high specificity to the toxin and to a lesser degree with its derivatives. The antibodies were raised in rabbits against STX and OA, rabbit anti-STX for PSP toxins and rabbit anti-OA for DSP toxins. The antibody binds to a second antibody against it raised in sheep in the case of rabbit anti-STX (sheep anti-rabbit), and goats in the case of rabbit anti-OA (goat anti rabbit). This second antibody is immobilised on the microtiter plate.

After incubation unbound toxins and toxin-enzyme conjugates were washed with washing buffer solution, and the microtiter plate was then dried thoroughly. The toxins bound to the toxin-enzyme conjugates were incubated for 30 minutes at room temperature with a substrate that changes colour in the presence of the enzyme attached to the toxin-enzyme conjugate. During incubation the microtiter plate was protected from direct sunlight. Incubation was stopped by adding a stop solution. Colour change was detected by measuring the change in absorbance at 450 nm using a Biotek ELx800 microtiter plate absorbance reader, and was inversely proportional to the amount of PSP or DSP toxins in the standard solution or sample.

2.4.2.3 Analysis of toxicity results

A commercial ELISA program , Gen5^{TM} fitted a 4-Parameter curve to the standard solutions (see below), creating a standard curve that was used to determine the toxin equivalents concentration of the samples, provided that the reading falls within the linear part of the standard curve, between the upper limit (UL) and lower limit (LL) (Fig. 2.2).

Toxin equivalents concentrations (ng.ml^{-1}) for the extracted samples were used to calculate those for the original mussel samples by multiplying the result by the dilution factors used in the assay, and were expressed as µg toxin eq.100 g^{-1}mussel:

$$\begin{gathered}\text{[mussel toxin](µg toxin eq.100 g}^{-1}\text{mussel)} = \\ \text{[sample toxin](ng.ml}^{-1}) \times \mathrm{D}_{assay} \times \mathrm{D}_{ext} \times \frac{1\,\mu\mathrm{g}}{1000\ \mathrm{ng}} \times \frac{\mathrm{V}_{ext}\ (\mathrm{ml})}{\mathrm{m}_{sample}\ (\mathrm{g})} \times 100\end{gathered}$$

Where

[mussel toxin] = final concentration of toxins in mussels

[sample toxin] = concentration of toxins in extracted samples of mussels

D_{assay}= dilution factors in assay (3X for PSP, 2X for DSP)

D_{ext}= dilution factors in extraction ($\geq$ 1000X)

V_{ext}= volume extracted

m_{sample}= mass of sample used in extraction

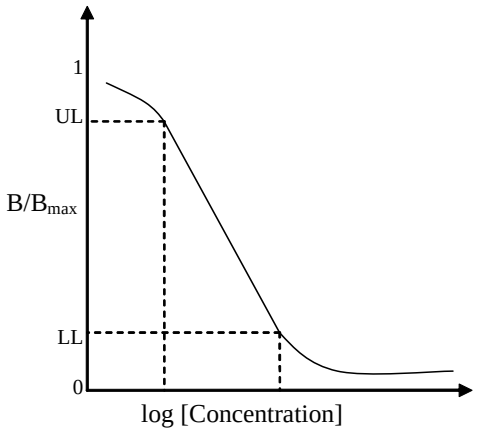


Figure 2.2: Standard curve for competitive binding assay, unknown concentrations are calculated by using readings from the linear part of the standard curve between the upper limit (UL) and the lower limit (LL), the formula for the 4-Parameter curve fitting is $y = \frac{(A-D)}{1+(\frac{x}{C})^b} + D$

Where

x =concentration

y =reading ($\frac{\mathrm{B}}{\mathrm{B}_{\max}}$)

A =estimated reference

B =slope factor

C =turning point

D =estimated blank

2.4.3 Receptor binding assay (RBA)

PSP toxins in the mussel samples were also quantified using a receptor binding assay (RBA), using the method described by Doucette *et al.* (1997), and modified by Powell & Doucette (1999). The RBA for PSP toxins is an *in vitro* assay, which quantifies the toxicity of samples using its toxic action, i.e. the binding of the toxin to neuroreceptors. Radioactively labelled saxitoxin (tritiated saxitoxin) competes with the unlabelled toxins in the standard solutions (saxitoxin) and extracted samples (saxitoxin and its derivatives) for binding sites on the neuroreceptors. The amount of tritiated saxitoxin ([^{3}H]STX) bound to the neuroreceptors is inversely proportional to the amount of toxin in the standard solution or sample and is measured using a scintillation counter. The unknown toxin concentration of samples were determined by comparison to a standard curve, which relates samples of a known toxin concentration with the amount of radiolabelled toxin bound.

2.4.3.1 Rat brain membrane preparation

The source of neuroreceptors for the RBA were rat brain membrane preparations (RBMP) from 6 week old male Sprague-Dawley rats (Zivic Laboratories, Inc., Pittsburgh, PA, USA; cat. no. TBR01.01). Rat brains were stored at -80°C before use. The cerebellum and medulla tissue was removed and the cerebral cortex was retained and buffered using a MOPS/choline chloride/PMSF buffer (pH 7.4), using 12.5 ml MOPS for each cortex. The buffer was prepared fresh by adding 1 ml 0.1 M PMSF (stored at -20°C) to 100 mM MOPS/choline chloride buffer, making it up to 1 L, and adjusting the pH to 7.4. During the preparation the rat brains were kept on ice. The buffered cerebral cortices were homogenised at 70% full speed (385 rpm), using at least 10 up and down strokes with a Caframo (Canada) homogeniser in a glass 30 ml homogenisation tube kept on ice. The pooled homogenate was centrifuged at 2 000 g for 15 minutes in a refrigerated centrifuge (4ºC). The supernatant was discarded and the homogenate pellet was resuspended in ~5 ml buffer, pooled and finally diluted with 10 ml buffer per brain. The pooled RBMP solution was thoroughly mixed using a Branson Sonifier 450 sonicator and 2 ml aliquots were dispensed into cryovials kept on ice, with mixing prior to each aliquot. These RBMP aliquots were stored at -80°C before use and are stable for up to 6 months.

2.4.3.2 Protein standard curve

In order to calculate the dilution of RBMP necessary to conduct the RBA the protein concentration in the RBMP was determined using a protein assay (Pierce Micro BCA Protein Assay, Reagent Kit #23225, Lot #F17214). The protein assay measures protein concentration directly by measuring the absorbance of a dye in the Working Reagent which changes colour after binding to protein. A linear standard curve was made from dilutions of bovine serum albumin (BSA), which reacts in a similar manner to the protein in the RBMP. One 2 ml aliquot of RBMP was thawed on ice overnight. The serial dilutions for the rat brain membrane preparation (RBMP) that were used are recorded in Table 2.1.

Table 2.1: Serial dilutions for rat brain membrane preparation (RBMP)

Dilution	Vial	RBMP (µl)	deionisedH_2O(µl)
1:10	A	100	900
1:20	B	500 of A	500
1:40	C	500 of B	500
1:80	D	500 of C	500
1:160	E	500 of D	500

A BSA stock solution of 2 mg.ml^{-1} was diluted 10X using deionised water. The diluted 200 µg.ml^{-1} BSA was stored in 500 µl aliquots at -20ºC.

Enough Working Reagent for all the samples to be analysed was prepared using the following formula:

V_{WR}(µl) = (# standards + # unknowns) x (# replicates) x 200 µl (volume of WR per sample)

The Working Reagent could be stored in a closed container at room temperature for a few days.

Standard solutions and the RBMP dilutions were added to the microplate wells in duplicate. The Working Reagent was then incubated together with the protein for 30 minutes at 37°C, was allowed to cool to room temperature and absorbance was read at a wavelength of 562 nm using a Biotek ELx800 microtiter plate absorbance reader. The standard curve created using the standard solutions of BSA was used to determine the correct dilution of RBMP in the receptor binding assay.

The standard curve for BSA was well described by a linear fit ($r^2 > 0.8$). The RBMP showed an absorbance reading within the correct range for the 1:20 dilution (ave. $A_{562} = 0.45$). The protein content was calculated by substituting into the equation for the line of best fit (given on the figure) and multiplying by the dilution factor of 20, and was calculated as 8 mg.ml^{-1}. This was then used to determine the dilution of RBMP needed in the RBA using the following formula:

$$\frac{\text{[actualRBMP]}(8\ \text{mg.ml}^{-1})}{\text{[final concentration needed in assay]}\ (0.5\ \text{mg.ml}^{-1})\ \text{x in assay dilution (1.5X)}} =\sim 11$$

Therefore in the assay 1 ml of RBMP was used for every 10 ml of MOPS buffer, this dilution fell within the recommended range of 1/8 - 1/12.

2.4.3.3 Working solution, standard curve and quality control check preparation

A 15 nM working solution of tritiated saxitoxin ([^{3}H]STX) was prepared on the day of the assay by adding 14 µl of stock [^{3}H]STX to 3.86 ml MOPS buffer, providing a final in assay concentration of 2.5 nM. An FDA STX dihydrochloride reference standard (268.8 µM or 100 µg.ml^{-1}) was used to make up a standard curve using a serial dilution series with 0.003 N hydrochloric acid as a diluent (Table 2.2), and could be stored at 4°C for up to a month. In assay the standards were diluted 1/6. An inter-assay calibration standard (or QC check) was prepared using part of the 1.8x10^{-8} M STX standard and 500 µl aliquots were kept frozen at -80°C. One of these was thawed and kept at 4°C for routine use, and would be stable in the thawed state for up to 1 month.

Table 2.2: Serial dilutions for receptor binding assay (RBA) standard curve

Dilution (stock) (M)	Dilution (in assay) (M)	Vial	268.8 µM STX diHCl (ml)	0.003 N HCl (ml)
$6x10^{-6}$	$1x10^{-6}$	A	0.1 of stock solution	4.38
$6x10^{-7}$	$1x10^{-7}$	B	0.5 of A	4.5
$1.8x10^{-7}$	$3x10^{-8}$	C	1.5 of B	3.5
$6x10^{-8}$	$1x10^{-8}$	D	0.5 of B	4.5
$1.8x10^{-8}$	$3x10^{-9}$	E	0.5 of C	4.5
$6x10^{-9}$	$1x10^{-9}$	F	0.5 of D	4.5
$6x10^{-10}$	$1x10^{-10}$	G	0.5 of F	4.5
$6x10^{-11}$	$1x10^{-11}$	H	0.5 of G	4.5
0	0	I	0	5

2.4.3.4 Extraction of mussel samples

Extract of the mussel homogenate for PSP toxins was prepared according to the AOAC (2000) method. Homogenised mussel samples (5 g) were measured into 15 ml conical tubes. The samples were extracted in 5 ml 0.1 N hydrochloric acid. pH was adjusted to between 3 - 4 by adding 1 N hydrochloric acid (in order to lower it) or 0.1 N sodium hydroxide (in order to raise it). The acidified mussel samples were placed in a boiling water bath for 5 minutes with caps loosened and allowed to cool to room temperature before readjusting the pH if necessary. The samples were made up to 10 ml with deionised water and after the mixture had settled, clear supernatant was then decanted and centrifuged at 3000 g for 10 minutes. The resulting supernatant was then filtered through a 0.45 µm nylon syringe filter. The filtered sample extract was stored at -20°C before analysis. Filtered sample extracts were thawed on ice overnight before the assay and diluted between 250X (for those with a low/intermediate toxin concentrations) and 10 000X (for those with a high toxin concentrations), with a range of three dilutions for each sample, to ensure that a result within the range of the standard curve was obtained.

2.4.3.5 RBA for PSP toxins

Standard solution, QC check solution or filtered sample extract (35 µl each) were added to the wells of a microplate in triplicate. This was followed by 35 µl of the tritiated saxitoxin working solution, and 140 µl of the RBMP, which was added last to ensure that reaction time was equal for all samples. The plate was then covered and incubated at 4°C for 1 hour. Unbound toxins were removed from the wells by a few cycles of vacuuming and washing the wells with cold MOPS buffer, using a DOA-P504-BN vacuum manifold (GAST, Mich, USA) with the vacuum strength adjusted until the wells were pulled to dryness in ~5 seconds. In order to ensure equal filtering pressure any empty wells were filled with MOPS buffer.

The plate was then placed into a counting cassette sealed on the underside with sealing tape. Optiphase scintillation cocktail (50 µl) was added and the top of the plate was also sealed with sealing tape. The plate was allowed to incubate for 30 minutes at room temperature before counting

in a Microbeta TriLux 1400 LSC & Luminescence microplate scintillation counter (Wallac/Perkin Elmer).

2.4.3.6 Analysis of toxicity results

A 4-Parameter curve was fitted using the Wallac Multicalc application provided by the manufacturer (Perkin-Elmer Wallac, Gaithersburg, MD), following the same parameters as the ELISA curve fitting for PSP toxins. The QC check had to be within $\pm$30% of 3 nM, and the readings that fell within the linear part of the curve (between UL = 0.7 and LL = 0.2) were used to calculate the concentration of the diluted sample extracts (in nM). These STX eq concentrations (nM) for the extracted samples were used to calculate those for the original mussel samples by multiplying the result by the dilution factors used in the assay and the molar weight of STX (ng.nmol^{-1}) (see below), and were expressed as µg STX eq.100g^{-1}mussel:

$$\text{[mussel toxin](µg STX eq.100 g}^{-1}\text{mussel)} = \text{[sample toxin](nM)} \times D_{assay} \times D_{ext} \times \frac{1\,\text{L}}{1000\ \text{ml}} \times \frac{327\ \text{ng}}{\text{nM}} \times \frac{1\,\mu\text{g}}{1000\ \text{ng}} \times \frac{V_{ext}\ \text{(ml)}}{m_{sample}\ \text{(g)}} \times 100$$

Where

[mussel toxin] = final concentration of toxins in mussels

[sample toxin] = concentration of toxins in extracted samples of mussels

D_{assay}= dilution factors in assay (6X)

D_{ext}= dilution factors in extraction ($\geq$ 250X)

V_{ext}= volume extracted

m_{sample}= mass of sample used in extraction

2.4.4 Mouse bioassay (MBA)

PSP toxins in the mussel samples were also quantified using the standard mouse bioassay (AOAC, 1990). Analyses were conducted by André Munian at the Council of Scientific and Industrial Research (CSIR) in Cape Town, South Africa. The assay involved acidic aqueous extraction of shellfish toxins followed by intraperitoneal injection of the extracted supernatant into mice. The time from initial injection to mouse death was recorded and used together with Sommer's Table to determine mouse units (MU). A mouse unit is defined as the amount of PSP toxin required to kill a ~20 g mouse within 15 minutes.

2.4.4.1 Extraction of mussel samples

Sub-samples of 100 g of mussel homogenate were dispensed into plastic screw-top bottles and stored at -20°C before analysis. Mussel homogenate was extracted using 0.1 N hydrochloric acid

followed by heating until boiling point. After the extract cooled to room temperature the pH was adjusted to between 2 - 4, by adding 5 N hydrochloric acid (in order to lower it) or 0.1 N sodium hydroxide (in order to raise it). This was allowed to settle until supernatant could be decanted free of homogenate, and if necessary it was centrifuged or filtered through paper to prevent blockage of a 26-gauge hypodermic needle.

2.4.4.2 MBA standardisation

The median death time was measured for groups of mice (at least ten) given 1 ml intraperitoneal injections of a standard solution of saxitoxin (1 μg.ml^{-1}), diluted until median death time was between 5-7 min. This was repeated for two 1 ml increments above and below the chosen dilution. For example if 10 ml is diluted with 25 ml to give correct death times, 10 ml diluted with 24 ml and 26 ml were also used. These were then used with Sommer's Table to convert death time to mouse units (with corrections for mice weighing < 19 g or > 21 g), and these mouse units were divided by the concentration of the diluted standard solution to give a conversion factor (CF). The CF is used to relate mouse units to toxin concentrations.

2.4.4.3 MBA for PSP toxins

For each test 1 ml of the acid extract was injected intraperitoneally into mice. If necessary this extract was diluted further until median death time was between 5 - 7 min, determined using 2 - 3 mice. For large dilutions the pH of the extract was checked and adjusted if necessary.

2.4.4.4 Analysis of toxicity results

Sommer's Table was used to determine mouse units (MU.ml^{-1}) from median death time (with correction for under or overweight mice). This was multiplied by the conversion factor (CF) calculated from the MBA standardisation and dilution factors used in the assay to determine toxicity in the mussel samples (μg STX eq.100g^{-1}mussel):

$$[\text{mussel toxin}](\mu\text{g STX eq.}100\ \text{g}^{-1}\text{mussel}) = \text{mouse units}(\text{MU.ml}^{-1}) \times \text{CF} \times \text{D}_{assay} \times \text{D}_{ext} \times 100$$

Where

[mussel toxin] = final concentration of toxins in mussels

mouse units = reading from Sommer's Table corresponding to median death time (with correction for weight)

CF = conversion factor

D_{assay}= dilution factors in assay (2X)

D_{ext}= dilution factors in extraction

2.4.5 Comparison of methods for detecting mussel toxicity

A Bland-Altman assessment for agreement (Bland & Altman, 1986) was used to compare the methods used to quantify mussel toxicity. Ratios of the measurements were used instead of differences for each pair of methods being compared, due to the observed increase in the magnitude of variability with an increase in toxin concentration. If the ratio of the two measurements was equal to 1 it indicated no difference between the methods being compared, while a ratio>1 indicated that the first method gave a larger measurement, and a ratio<1 indicated that the second method gave a larger measurement. The 95% limits of agreement is defined as mean bias ±1.96 SD.

Chapter 3

Results

3.1 The physical environment and toxigenic phytoplankton species

The survey period (20 March - 11 April 2007) was characterised by two upwelling events, at which time the surface water temperature dropped to 11ºC (Fig. 3.1.A). The first upwelling event (centred around 21 March 2007) was followed by a short period of relaxation where the surface water temperature rose to 14°C, whereas the second upwelling event (centred around 27 March 2007) was followed by a longer relaxation period resulting in highly stratified conditions where the surface water temperature rose to 18°C. Each upwelling event was followed by an increase in the density of phytoplankton, reflected by an increase in the concentration of chlorophyll-*a*, reaching a subsurface maximum of 38 mg.m^{-3} after the first upwelling event and 63 mg.m^{-3} after the second upwelling event (Fig. 3.1.B).

The survey period was initially dominated by the toxigenic dinoflagellate *A. catenella*, whose cell concentrations reached a subsurface maximum of 6.1x10^5 cells.L^{-1}before declining during the first upwelling event (Fig. 3.2.A). In the period of relaxation following the first upwelling event, the dominant *A. catenella* population was replaced by a diatom dominated bloom, including toxigenic diatoms of the *Pseudo-nitzschia* spp., reaching a subsurface maximum of 9.5x10^5 cells.L^{-1}. This was later replaced with a mixed assemblage of phytoplankton including toxigenic dinoflagellates of the *Dinophysis* spp., mostly *D. acuminata*, but also including *D. fortii*, *D. hastata* and *D. rotundata* (Fig. 3.2.B & C), reaching a subsurface maximum of 8.3x10^4 cells.L^{-1}. The second upwelling event resulted in phytoplankton cell concentrations decreasing sharply, with a corresponding drop in chlorophyll-*a* concentration. During the extended period of warming following the second upwelling event, diatoms initially dominated the phytoplankton assemblage, with toxigenic *Pseudo-nitzschia* spp. reaching a subsurface maximum of 1.2x10^6 cells.L^{-1}. As stratification intensified the diatom population was replaced by a mixed assemblage of phytoplankton, including *Dinophysis* spp. In the late part of the survey when the water was highly stratified and nutrients were depleted small flagellates were observed to dominate the phytoplankton assemblage. For all toxigenic phytoplankton the highest densities were recorded 5 m below the surface and occasionally at the surface.

3.2 Paralytic shellfish poisoning toxins

3.2.1 Cell-toxin quota for *Alexandrium catenella*

PSP toxin concentrations as extracted from filtered plankton samples corresponded approximately with cell concentrations of *A. catenella* (Fig. 3.3). *A. catenella* concentrations reached a subsurface maximum of 6.1x10^5 cells.L^{-1} on 22 March whereas PSP toxin concentrations reached a subsurface maximum of 4.7x10^7 pg STX eq.L^{-1} on 21 March. *A. catenella* cell density was plotted against the concentration of particulate PSP toxins (Fig. 3.4). While the presence of *A. catenella* generally coincided with particulate PSP toxins it was highly variable, as demonstrated by the low r^2 value (0.47). It was however a statistically significant relationship ($p < 0.05$). The average cell-toxin quota determined from this relationship for the survey period was 39.4 pg STX eq.$cell^{-1}$.

3.2.2 *Alexandrium catenella* toxin profile and biotransformation in the mussel

Individual PSP toxin values quantified using hydrophilic interaction liquid chromatography linked with tandem mass spectrometry (HILIC-MS/MS) were used to determine the PSP toxin profile of *A. catenella*, and the composition of accumulated toxins by the mussel *C. meridionalis* (Fig. 3.5).

The toxin profile of *A. catenella* was relatively conserved (10% STX; 60% NEO; 25% C1,2; trace amounts of GTX2,3; dcGTX2,3; B1), consistently showing a large proportion (70%) of the more toxic carbamate derivatives, especially neosaxitoxin (NEO).

Several toxin derivatives varied significantly between *A. catenella* and the mussel (STX, NEO, and C1,2) providing an indication of biotransformation. The composition of PSP toxins in the mussel *C. meridionalis* (27% STX; 18% NEO; 50% C1,2; trace amounts of dcSTX; GTX1,2,3; B1) demonstrated a significantly higher proportion of the less toxic N-sulfocarbamoyl derivatives C1,2 (55%) and the most potent toxin, saxitoxin (STX), as well as a decrease of the proportion of the highly toxic carbamate derivative neosaxitoxin (NEO).

3.2.3 Uptake and clearance of PSP toxins by the mussel

PSP toxin concentrations in the mussel remained fairly constant during the initial part of the survey period until 30 March (Fig 3.6), indicating that there was no net uptake or clearance of toxins during this period. The average toxin concentration calculated from all the methods used estimated a range of 4-7x10^3 µg STX eq.$100g^{-1}$ mussel, well over the regulatory limit of 80 µg STX eq.$100g^{-1}$ mussel. The high PSP toxin concentration extended for a week following the

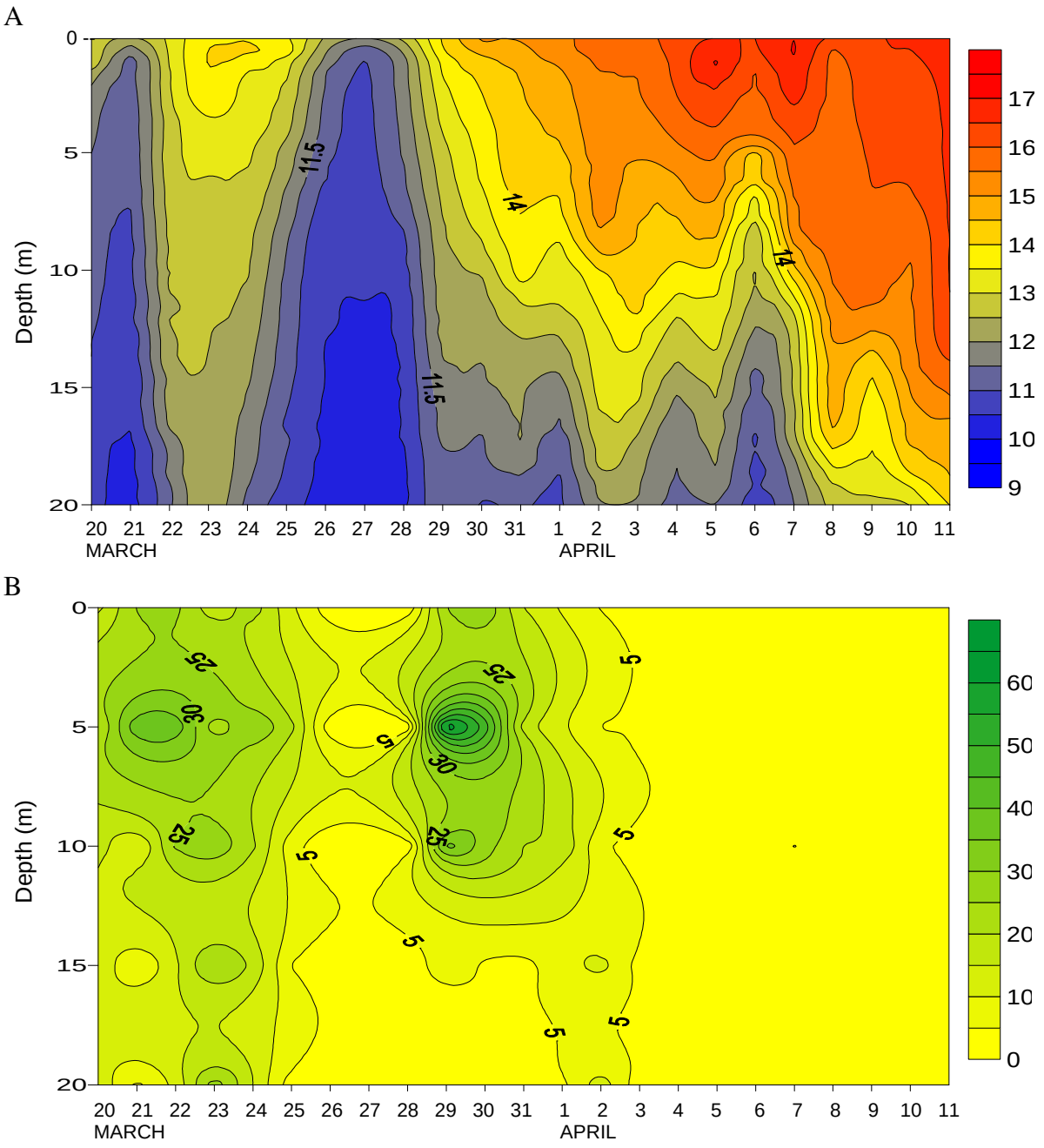


Figure 3.1: A daily time series of (A) temperature (°C) and (B) chlorophyll-*a* (mg.m^{-3}) at the mooring off Lambert's Bay from the 20 March - 11 April 2007. The contour maps were created using Surfer v8.01.

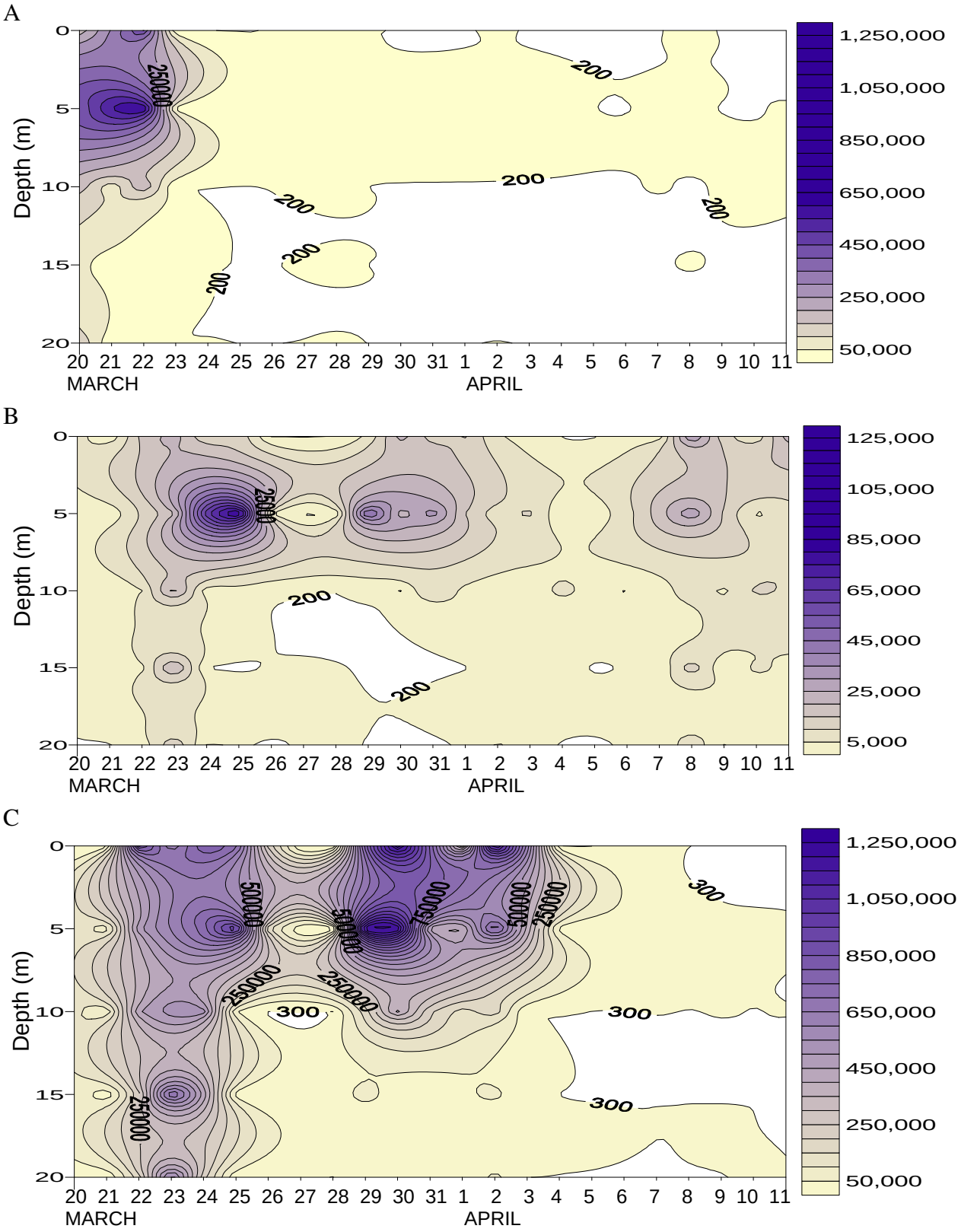


Figure 3.2: A daily time series of the toxigenic phytoplankton species (A) *Alexandrium catenella*, (B) *Dinophysis* spp., and (C) *Pseudo-nitzschia* spp., at the mooring off Lambert's Bay from 20 March - 11 April 2007. The contour maps were created using Surfer v8.01.

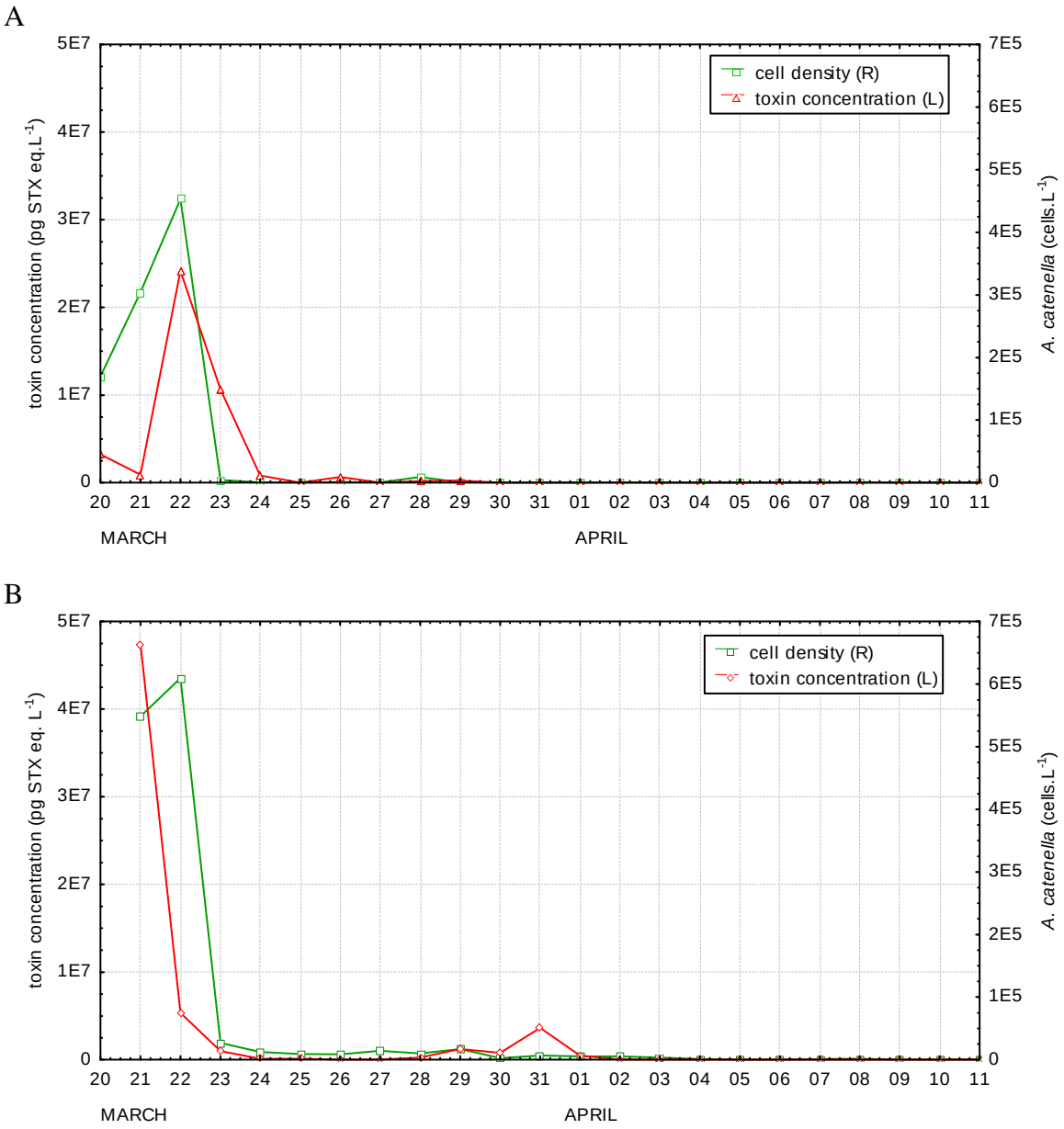


Figure 3.3: A daily time series of *Alexandrium catenella* cell concentrations (cells.L^{-1}) and particulate PSP toxin concentrations (pg STX eq.L^{-1}) detected in seawater samples at (A) 0 m and (B) 5 m, from 20 March - 11 April 2007.

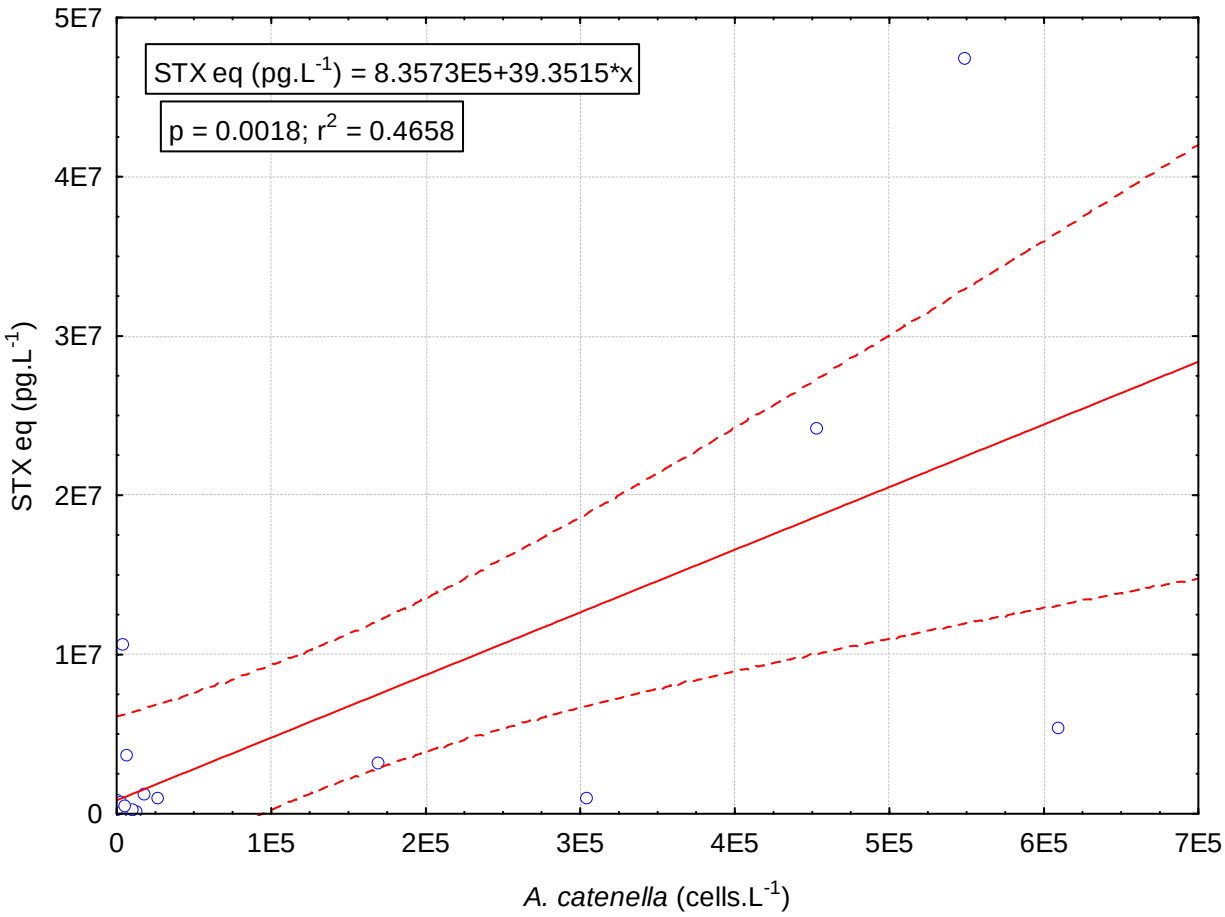
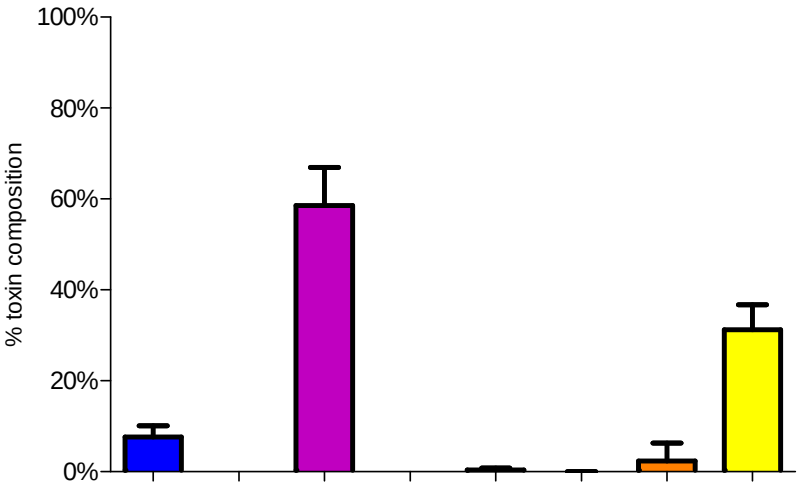
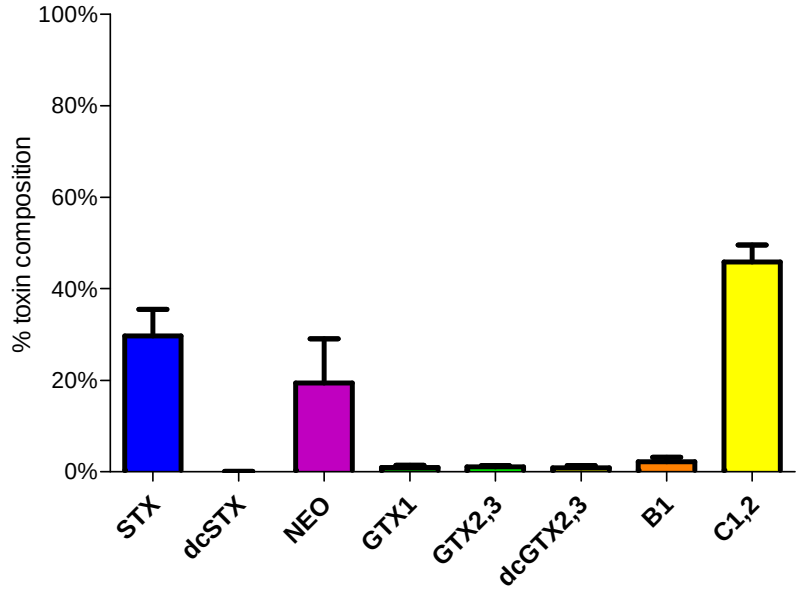


Figure 3.4: *Alexandrium catenella* cell concentrations (cells.L^{-1}) plotted against particulate PSP toxin concentrations (pg STX eq.L^{-1}). A linear regression was fitted using Statistica ver 9.0., the dotted lines represent the 95 % confidence bands.

A



B



Bonferroni Post Hoc Test		
Toxin	**P-value**	**Significant/Non-significant**
STX	<0.001	s
dcSTX	>0.05	ns
NEO	<0.001	s
GTX1	>0.05	ns
GTX2,3	<0.001	ns
dcGTX2,3	<0.001	ns
B1	<0.001	ns
C1,2	>0.05	s

Figure 3.5: (A) The toxin profile of *Alexandrium catenella* compared to (B) the composition of PSP toxins in the mussel *Choromytilus meridionalis* with standard error bars created using GraphPad Prism v5. A Bonferroni post-hoc test was used to test for significant differences between the individual toxins in *A. catenella* and *C. meridionalis*.

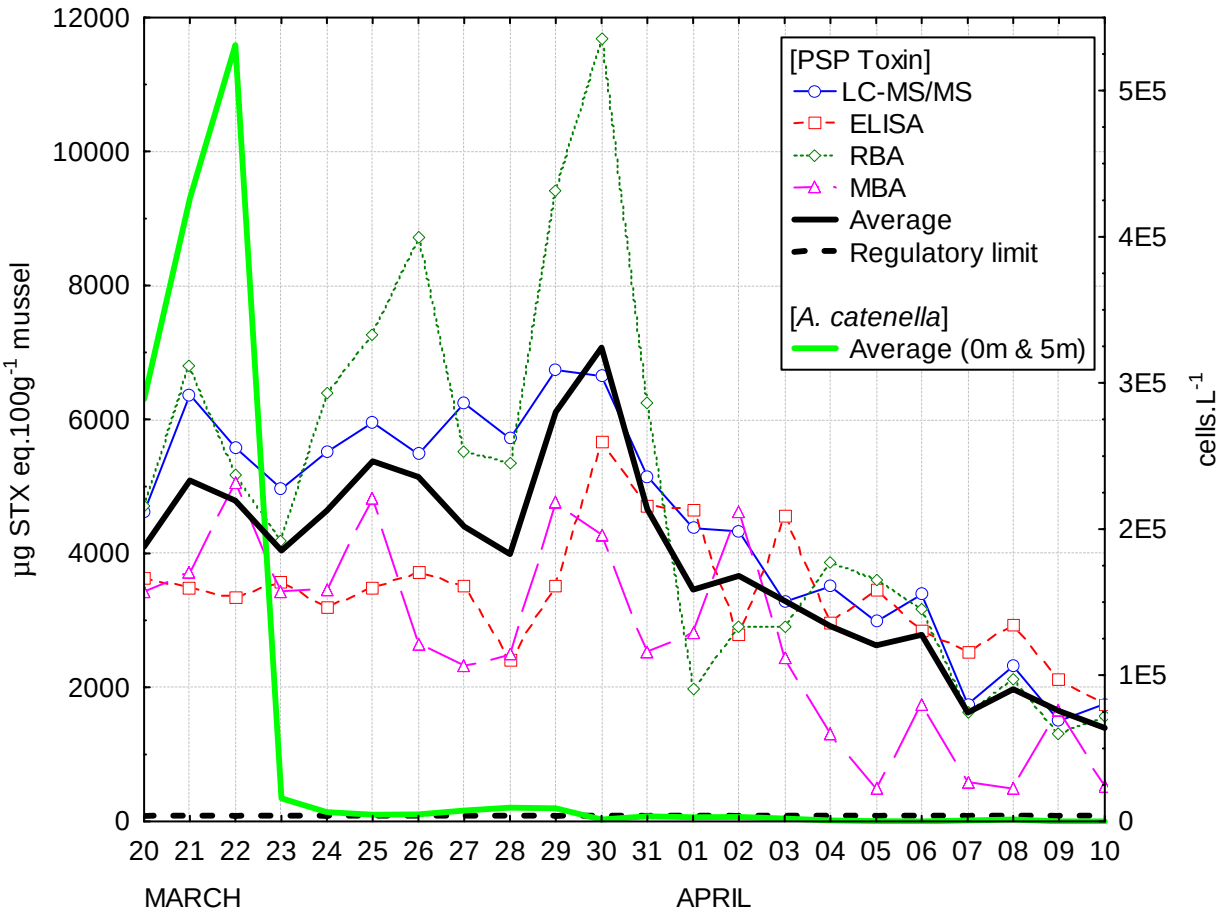


Figure 3.6: A daily time series of PSP toxin concentration (µg STX eq.100g^{-1} mussel) for the mussel *Choromytilus meridionalis*, measured using hydrophilic interaction liquid chromatography linked with tandem mass spectroscopy (HILIC-MS/MS), an enzyme-linked immunosorbent assay (ELISA), a receptor binding assay (RBA) and the mouse bioassay (MBA). The average toxin concentration for all of the methods as well as cell concentrations (cells.L^{-1}) of *Alexandrium catenella* at 0 m and 5 m are included. The regulatory limit for PSP toxin concentration in shellfish is 80 µg STX eq.100g^{-1} mussel.

decline of the bloom of *A. catenella* on 23 March, reaching a maximum of 7x10^3 µg STX eq.100g^{-1} mussel on 30 March. Following 30 March an initial rapid clearance of PSP toxins was followed by a more gradual clearance until the end of the survey period, never falling below the regulatory limit.

3.2.3.1 Comparison of methods used to quantify PSP toxins in the mussel

Bland-Altman plots of ratios vs. the average (Fig. 3.7) of results were used to compare pairs of the four methods used to quantify PSP toxin concentrations. A mean ratio value approaching 1,a small standard deviation, and a high correlation co-efficient would imply a good agreement between the two methods compared in each pair.

The hydrophilic interaction liquid chromatography linked with tandem mass spectrometry (HILIC-MS/MS) agreed well with the enzyme-linked immunosorbent assay (ELISA) (Fig. 3.7.A) and the receptor binding assay (RBA) (Fig. 3.7.B) but not the mouse bioassay (MBA) (Fig. 3.7.C). It

showed the best agreement with the receptor binding assay with the mean ratio value close to 1 as well as a small standard deviation (mean 1.03, sd 0.33) and also had a high correlation co-efficient (r^2= 0.73). Agreement with ELISA was also relatively good (mean 1.3, sd 0.46) however the correlation co-efficient was low between these two methods (r^2= 0.28). The agreement with the MBA was the poorest (mean 2.1, sd 1.25), however these methods showed a high level of correlation (r^2= 0.63).

The ELISA also compared well to the RBA (Fig. 3.7.D), but not the MBA (Fig. 3.7.E). It showed a better agreement with the RBA than with the HILIC-MS/MS (mean 0.9, sd 0.5) but also with a fairly low correlation co-efficient (r^2= 0.31). The agreement with the MBA was once again poorest (mean 1.9, sd 1.7) and had the lowest correlation co-efficient (r^2= 0.17).

The RBA also showed its poorest agreement with the MBA (Fig. 3.7.F), (mean 2.2, sd 1.5) and had a fairly low correlation co-efficient (r^2= 0.39).

3.2.3.2 Depuration of PSP toxins in the mussel

In the mussel *C. meridionalis*, concentrations of all PSP toxins except the decarbamoyl derivatives (dcSTX and dcGTX2,3) decreased significantly after the 30 March (Fig. 3.8). A non-linear regression analysis was used to calculate the best-fit models. The model that best described the depuration of PSP toxins in the mussel *C. meridionalis* was a one-phase exponential decay model, where rapid initial depuration was followed by slower depuration.

$C = C_0.e^{-k.t}$

Where

C = concentration of toxins

C_0 = initial concentration of toxins

k = rate constant

t = time

The rates of depuration were fastest for the most potent toxins, the carbamate derivatives STX and NEO, with a rate constant k value of 0.1 and 0.2 day^{-1} respectively. The other carbamate derivatives GTX1,2,3 as well as the least potent N-sulfocarbamoyl derivatives B1 and C1,2 showed an intermediate rate of depuration, with k values ranging from 0.06 - 0.09 day^{-1}. The decarbamoyl derivatives had a rate of depuration that was not significantly different from 0, with k values < 0.01 day^{-1}.

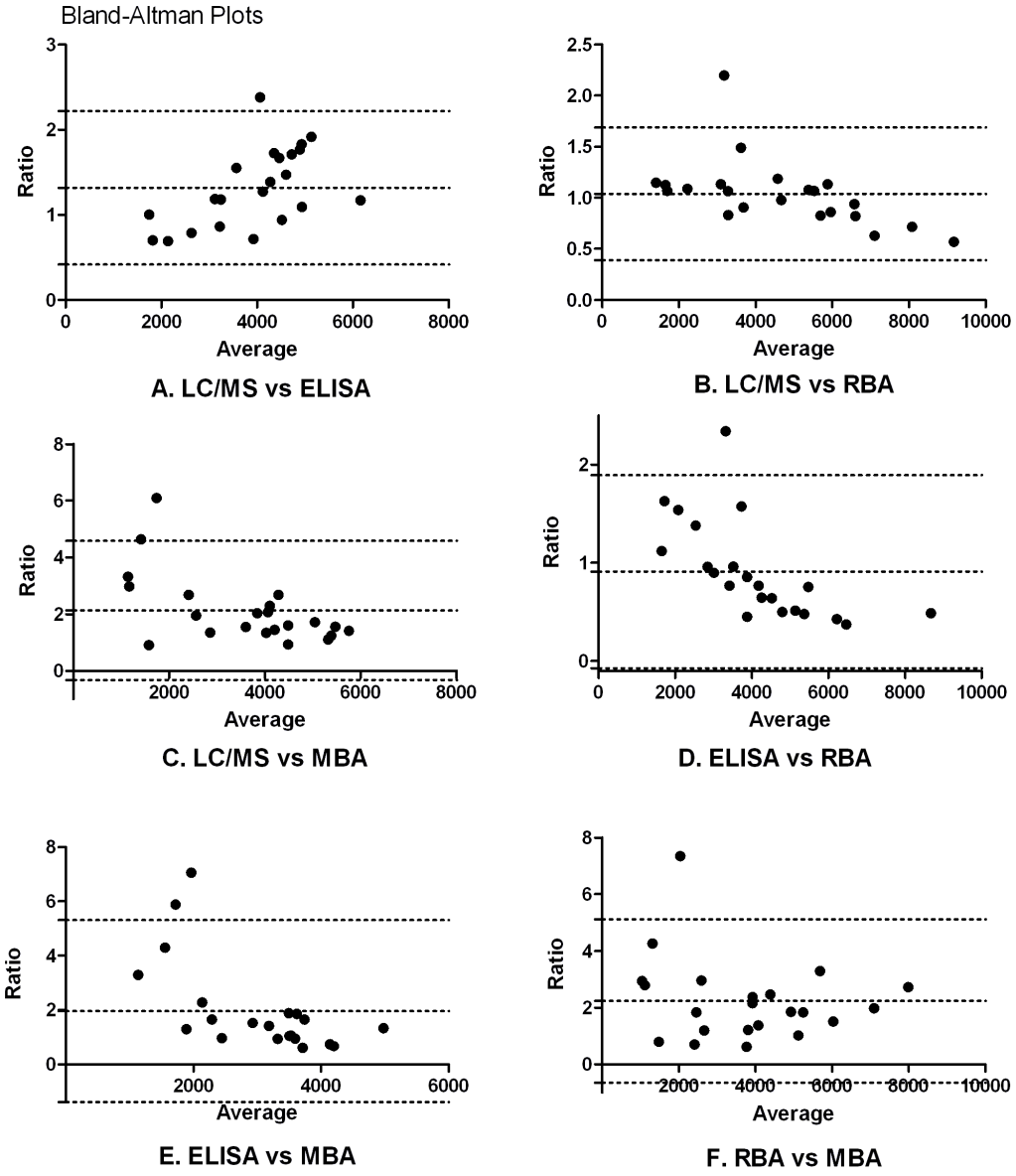


Figure 3.7: Bland-Altman plots (ratio vs. average) of the four methods used to measure paralytic shellfish poisoning (PSP) toxins in the mussel *Choromytilus meridionalis*, including hydrophilic liquid chromatography linked with tandem mass spectrometry (HILC-MS/MS), an enzyme-linked immunosorbent assay (ELISA), a receptor binding assay (RBA) and a mouse bioassay (MBA). Plots were created using GraphPad Prism ver. 5, dotted lines represent the 95% limits of agreement (mean bias $\pm$1.96 SD).

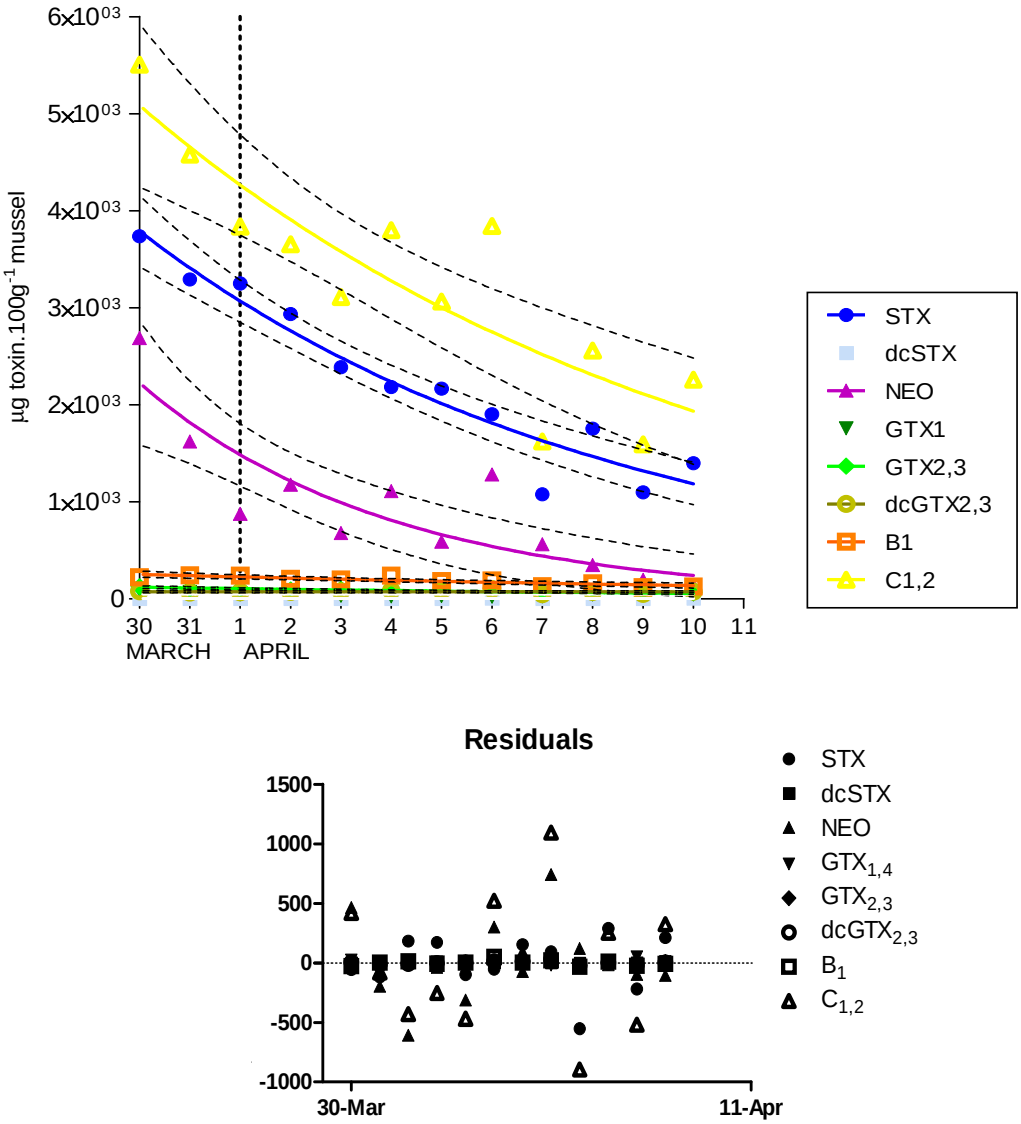


Figure 3.8: A daily time series showing the depuration of PSP toxins from the mussel *Choromytilus meridionalis* after *Alexandrium catenella* was only present at relatively low concentrations, from the 30 March - 11 April 2007. The best model fit for the depuration of these toxins was a one-phase exponential decay curve, created using GraphPad Prism v5, dotted lines represent 95% confidence bands. Residual plots are shown above and further statistical analyses are recorded in Table 1 on page 83 (Appendix A).

3.3 Diarrhetic shellfish poisoning toxins

3.3.1 Cell-toxin quota for *Dinophysis* spp.

Cell concentrations of *Dinophysis* spp. corresponded approximately to DSP toxin concentrations in the particulate fraction of seawater samples (Fig. 3.9). Several *Dinophysis* spp. were observed, mostly *D. acuminata* with some *D. hastata, D. fortii* and *D. rotundata*. The *Dinophysis* spp. cell concentrations reached a subsurface maximum of $8.3x10^4$ cells.L^{-1}on 25 March, whereas total DSP toxin concentrations reached a sub-surface maximum of $1.17x10^4$ pg OA eq.L^{-1} on 24 March. *Dinophysis* spp. cell density was plotted against the concentration of particulate DSP toxin concentrations (Fig. 3.10). *Dinophysis* spp. generally coincided with DSP toxins in the particulate fraction, however there was a great deal of variability in the relationship as demonstrated by the low r^2 value (0.32) but was nevertheless statistically significant ($p < 0.05$). The average cell-toxin quota determined from this relationship for the survey period was 0.07 pg OA.$cell^{-1}$.

3.3.2 *Dinophysis* spp. toxin profile and biotransformation in the mussel

Individual DSP toxin values quantified using liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) were used to determine the DSP toxin profile of the *Dinophysis* spp., and the composition of these toxins in the mussel *C. meridionalis* (Fig. 3.11). The toxin profile for the *Dinophysis* spp. (95% OA; 4% DTX1; trace amounts of PTX2) was relatively conserved and with a consistently large proportion (95%) of the more toxic parent compound, okadaic acid (OA).

The toxin composition of the mussel *C. meridionalis* (77% OA; 18% DTX1; trace amounts of PTX2; PTX2sa) showed a significantly higher proportion of the less toxic dinophysistoxin derivative DTX1 (~20% compared to 5%). Low concentrations of pectenotoxins (PTX2 and PTX2sa) were measured. Toxins of the okadaic acid group (OA and DTX1) showed a significant difference in their relative proportions between the *Dinophysis* spp. and the mussel samples, indicating biotransformation of toxins by the mussel.

3.3.3 Uptake and clearance of DSP toxins by the mussel

DSP toxin concentrations in the mussel *C. meridionalis* gradually increased during the initial part of the survey, from very low concentrations to above the regulatory limit of 16 µg OA eq.$100g^{-1}$mussel after the 3 April. After 3 April, nine days after toxic *Dinophysis* spp. had reached their maximum density on the 25 March, DSP toxin concentration in the mussel increased more rapidly, reaching a maximum of on 9 April of 43 µg OA eq.$100g^{-1}$mussel, calculated using the average for the two methods used.

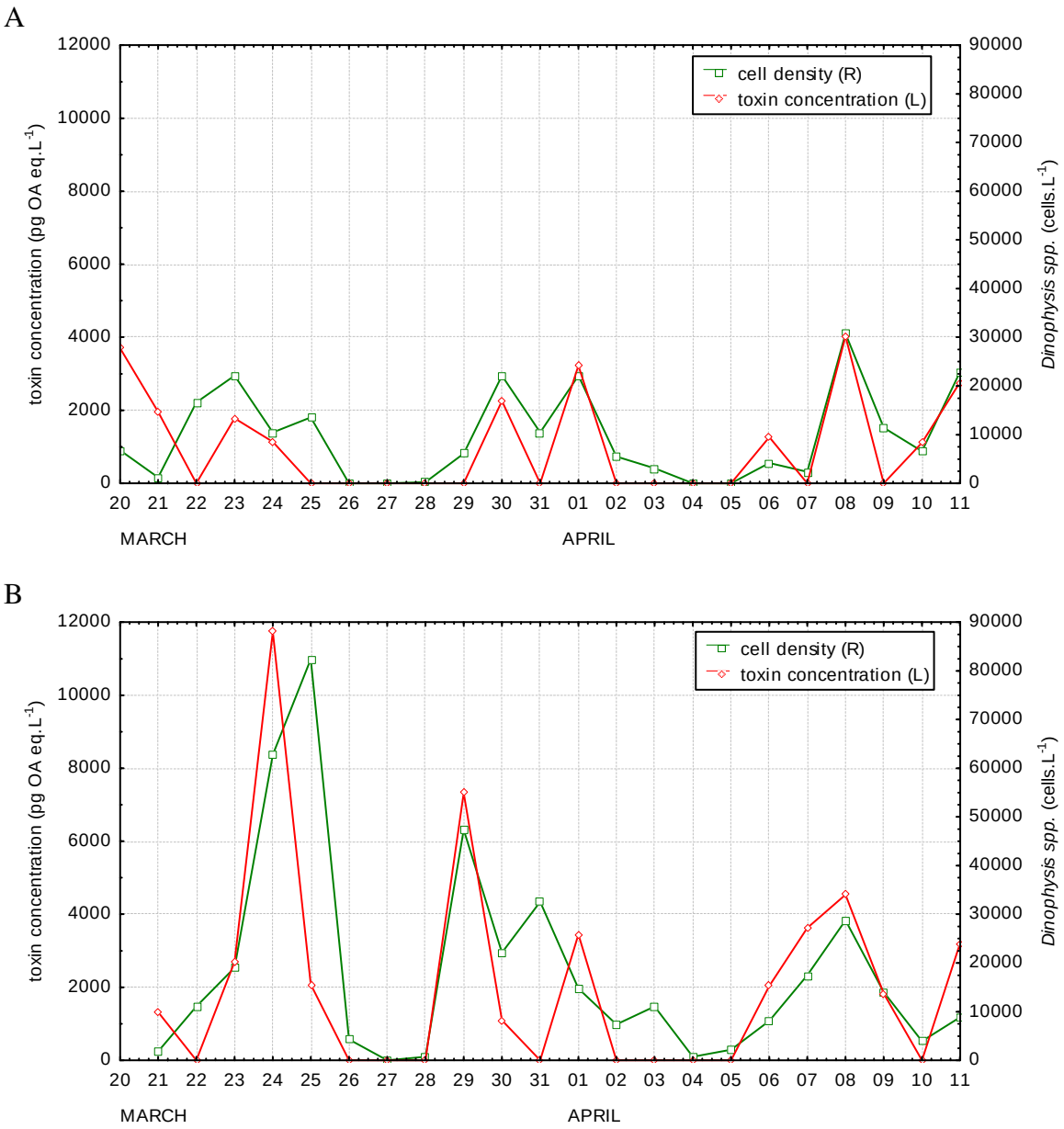


Figure 3.9: A daily time series of *Dinophysis* spp. (mainly *D. acuminata*, with some *D. fortii*, *D. hastata* and *D. rotundata* present) cell concentrations (cells.L^{-1}) and particulate DSP toxin concentration (pg OA eq.L^{-1}) in seawater samples at (A) 0 m and (B) 5 m from 20 March - 11 April 2007.

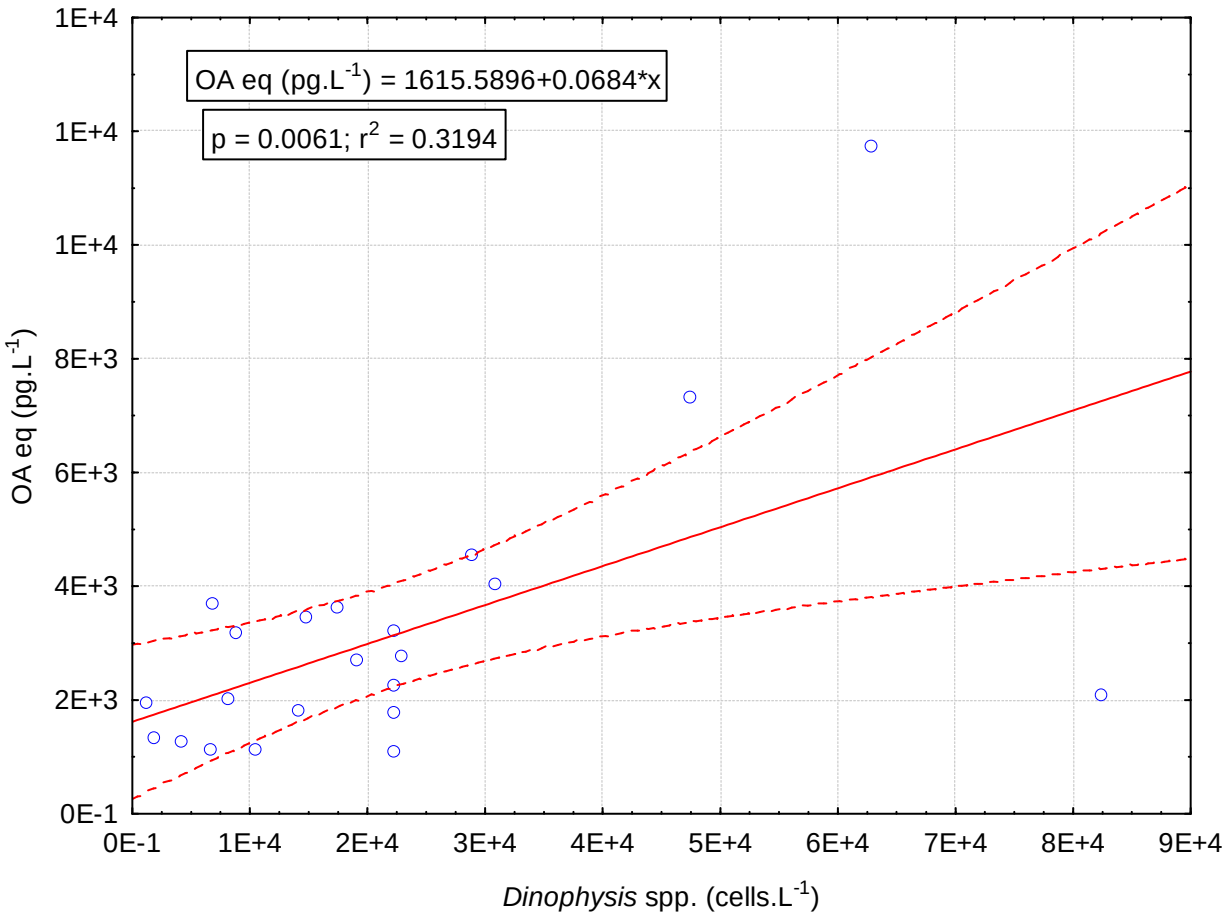
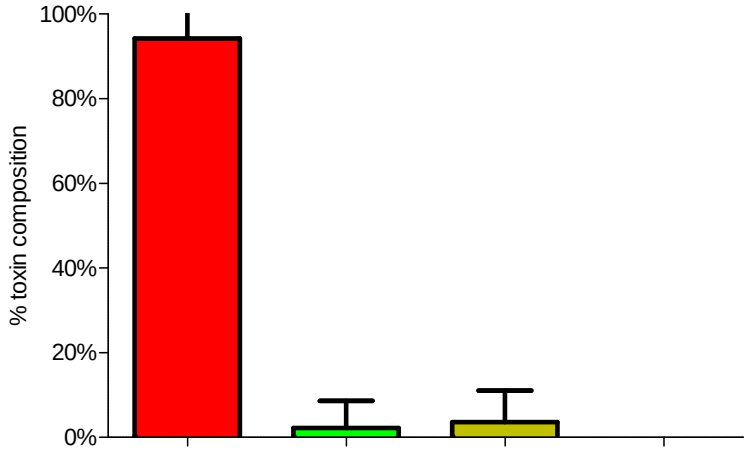
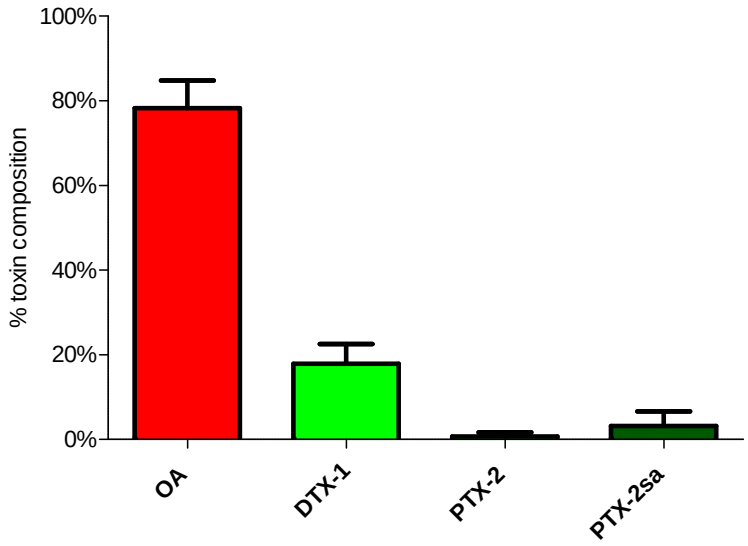


Figure 3.10: *Dinophysis* spp. cell concentrations (cells.L^{-1}) plotted against particulate DSP toxin concentrations (pg OA eq.L^{-1}). A linear regression was fitted using Statistica ver 9.0, the dotted lines represent the 95 % confidence bands.

A



B



Bonferroni Post Hoc Test		
Toxin	**P-value**	**Significant/Non-significant**
OA	<0.001	s
DTX-1	<0.001	s
PTX-2	>0.05	ns
PTX-2sa	>0.05	ns

Figure 3.11: (A) Toxin profile for the *Dinophysis* spp. (mainly *D. acuminata*, with some *D. fortii*, *D. hastata* and *D. rotundata* present) and (B) the composition of DSP toxins in the mussel *Choromytilus meridionalis* with standard error bars (created using GraphPad Prism v5). A Bonferroni post-hoc test was used to test for significant differences between individual toxins in *Dinophysis* spp. and *C. meridionalis*.

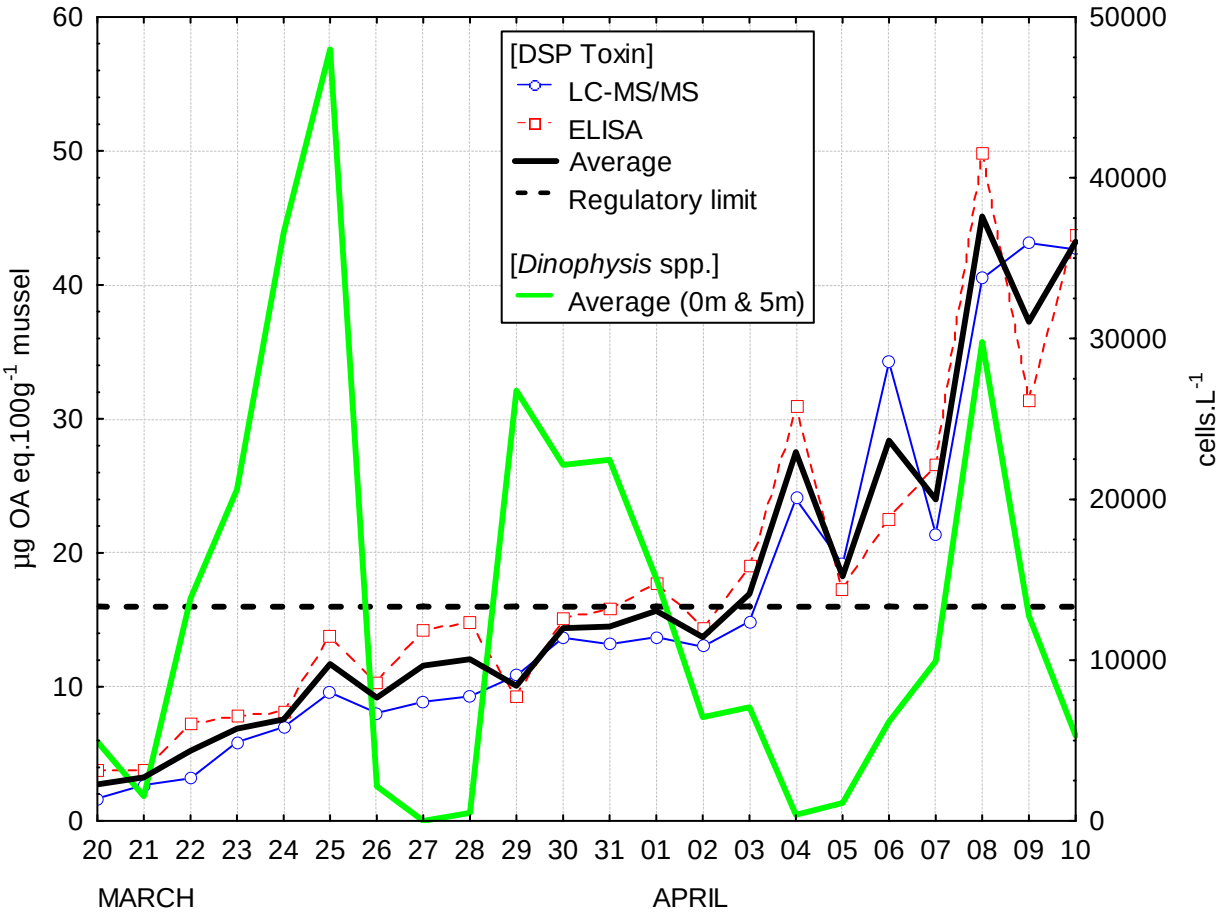


Figure 3.12: DSP toxin concentration (µg OA eq.100g^{-1} mussel) for the mussel *Choromytilus meridionalis*, measured using liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) and an enzyme-linked immunosorbent assay (ELISA). The average cell concentrations (cells.L^{-1}) of *Dinophysis* spp. at 0 m and 5 m was included. The regulatory limit for DSP toxins in shellfish is 16 µg OA eq.100g^{-1} mussel.

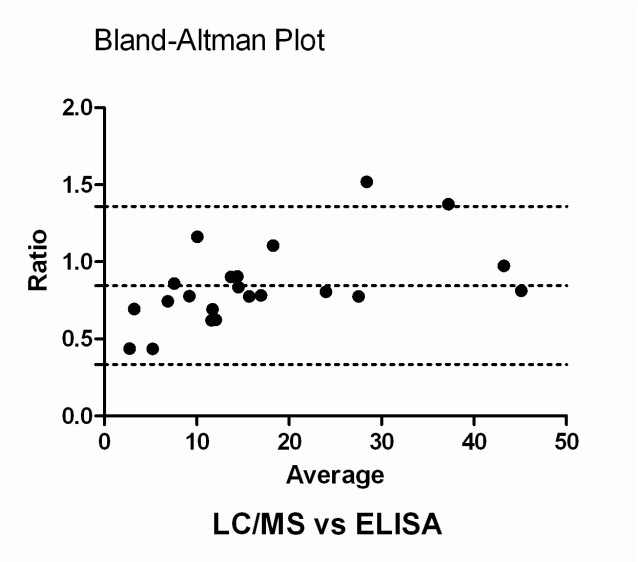


Figure 3.13: Bland-Altman plot (ratio vs. average) of the two methods used to measure paralytic shellfish poisoning (PSP) toxins in the mussel *Choromytilus meridionalis*, liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) and an enzyme-linked immunosorbent assay (ELISA). Plots were created using GraphPad Prism ver. 5, dotted lines represent the 95% limits of agreement (mean bias $\pm$1.96 SD).

3.3.3.1 Comparison of methods used to quantify DSP toxins in the mussel

For the two methods used to measure DSP toxicity, LC-MS/MS and ELISA, the Bland-Altman plot showed a good agreement between the results from the two methods (Fig. 3.13). The mean of the ratio of results given by the two methods approached 1 (mean 0.8), the standard deviation was small (sd 0.3), and the correlation co-efficient was high (r^2= 0.86).

3.4 Amnesic shellfish poisoning toxins

3.4.1 Cell-toxin quota for *Pseudo-nitzschia* spp.

Toxigenic diatoms were only identified down to genus level. Total *Pseudo-nitzschia* spp. density peaked three times, the highest peak on 30 March with a maximum of 1.2x10^6 cells.L^{-1} both at the surface and subsurface. *Pseudo-nitzschia* spp. and particulate domoic acid (DA) concentrations in the seawater samples corresponded approximately, with DA toxins reaching a sub-surface maximum concentration of 4.6x10^5 pg DA.L^{-1} on 30 March (Fig. 3.14). *Pseudo-nitzschia* spp. cell density is plotted against ASP toxin concentrations in Fig. 3.15. *Pseudo-nitzschia* spp. coincided with DA detected in the particulate fraction of seawater samples, the high variability in the cell-toxin quota of *Pseudo-nitzschia* spp. was demonstrated by a low r^2 value (0.37) but the relationship was statistically significant ($p < 0.05$). The average cell-toxin quota for the survey period was 0.19 pg DA.$cell^{-1}$.

Despite the presence of domoic acid in the particulate fraction of seawater samples, no domoic

acid was detected in the mussel samples.

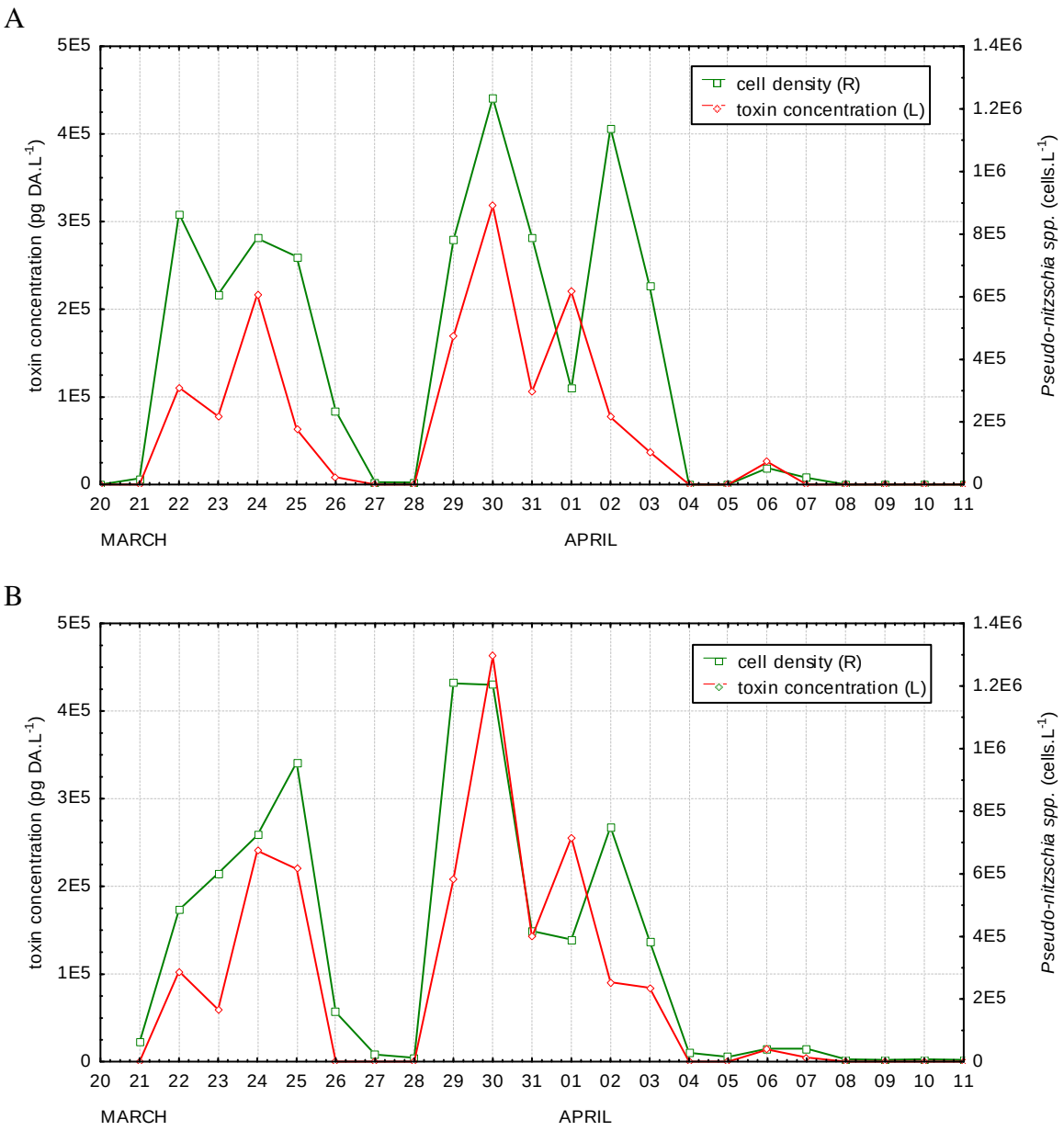


Figure 3.14: A daily time series of *Pseudo-nitzschia* spp. cell concentrations (cells.L^{-1}) and particulate ASP toxin concentrations (pg DA.L^{-1}) in seawater samples at (A) 0 m and (B) 5 m from 20 March - 11 April 2007.

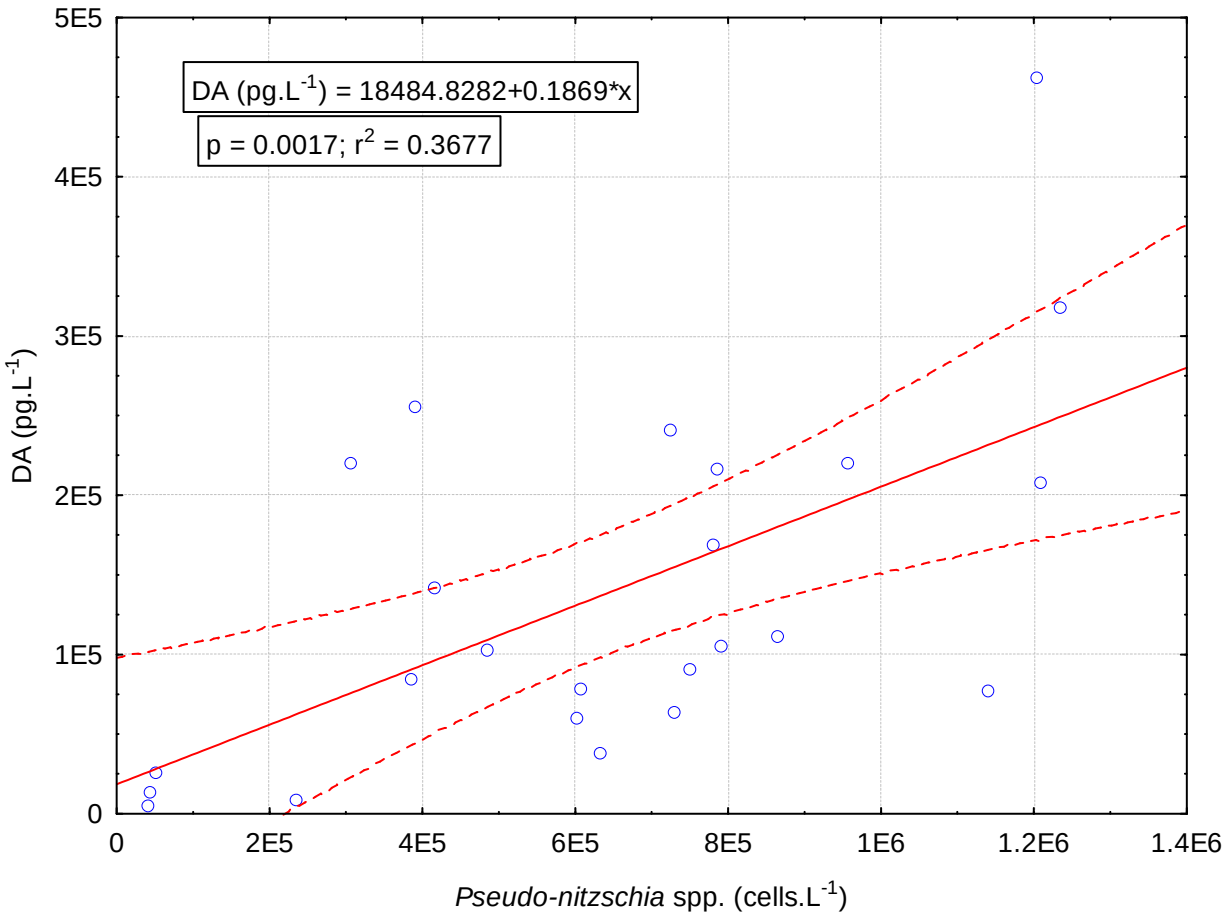


Figure 3.15: *Pseudo-nitzschia* spp. cell concentrations (cells.L^{-1}) plotted against particulate ASP toxin concentrations (pg DA.L^{-1}). A linear regression was fitted using Statistica ver 9.0, the dotted lines represent the 95 % confidence bands.

Chapter 4

Discussion

4.1 Upwelling-relaxation cycles and toxigenic phytoplankton species

Chlorophyll-*a* readings provided an indication of overall phytoplankton biomass in response to upwelling-relaxation cycles. Two upwelling events occurred during the survey period, the first of which was followed by a brief relaxation period and the second by a much longer relaxation period. The concentration of chlorophyll-*a* decreased during both upwelling events, especially during the second upwelling event, indicating a drop in phytoplankton biomass. This is expected, as newly upwelled water is nutrient-rich, cool, turbulent, and with low phytoplankton biomass. Chlorophyll-*a* concentrations increased as water warmed during relaxation periods, as phytoplankton had access to both light and nutrients. Chlorophyll-*a* concentrations decreased once again during the second and longer period of relaxation, as nutrients necessary for phytoplankton growth and cell division were depleted. The subsurface maxima of 38 and 63 mg chl-$a.m^{-3}$ are characteristic of chlorophyll concentrations for the southern Benguela in stratified water following upwelling events (Barlow, 1982).

Upwelling and relaxation cycles result in different environmental conditions, which favour different groups of phytoplankton (Margalef, 1978), as demonstrated by the toxigenic phytoplankton present in this survey. There were two upwelling episodes during this survey which changed the toxigenic phytoplankton species assemblage present in the water column.

The first upwelling event reset the phytoplankton species assemblage, which changed from dinoflagellate dominated (*Alexandrium catenella*) to diatom-dominated (*Pseudo-nitzschia* spp.). The dominance of *A. catenella* in the phytoplankton assemblage is not typical of all systems (Anderson, 1998), and high biomass is atypical of newly upwelled water. However *Alexandrium*-dominated blooms are common off the west coast of South Africa, especially of *A. catenella* (Pitcher & Calder, 2000), with other species such as *A. minutum* occasionally also reaching high biomass in the southern Benguela (Pitcher *et al.*, 2007). The rapid decline in *A. catenella* in the first upwelling event can probably be attributed to physical processes, as upwelling is associated with the

advection of surface waters and high biomass offshore and the introduction of phytoplankton-poor deeper water. The well-mixed nutrient-rich water immediately following the first upwelling event would favour non-motile diatoms such as *Pseudo-nitzschia* adapted to using high concentrations of nutrients efficiently (Bates *et al.*, 1998; Seeyave *et al.* 2009).

During the relaxation period following the first upwelling event the diatom-dominated bloom was replaced by a mixed phytoplankton assemblage, including toxigenic *Dinophysis* spp.. During relaxation periods the water became warmer and more stratified, with nutrients becoming depleted in the euphotic zone, favouring motile dinoflagellates such as *Dinophysis* that can source nutrients from depth and are adapted to more nutrient poor conditions (Seeyave *et al.*, 2009). The relatively low cell concentrations of *Dinophysis* spp. in a mixed phytoplankton species assemblage is typical of *Dinophysis* blooms (Maestrini, 1998).

The second upwelling event reset the phytoplankton species assemblage again, which was once more dominated by diatoms (*Pseudo-nitzschia* spp.), which were later replaced by the mixed phytoplankton assemblage including toxigenic *Dinophysis* spp., and later by small flagellates. Highly stratified and nutrient depleted water favours small flagellates, which have lower nutrient requirements and are motile and therefore able to source nutrients from depth.

4.2 Paralytic shellfish poisoning toxins

4.2.1 Cell-toxin quota for *Alexandrium catenella*

The cell-toxin quota for *A. catenella* showed a statistically significant positive relationship between PSP toxin concentration in the particulate fraction of the water column and *A. catenella* cell concentration, but with a great deal of variability. Low temperatures would be associated with higher cell-toxin quotas, due to the the associated decrease in metabolic rates and cell division (Cembella, 1998).The most likely explanations for variability in cell-toxin quota over the short period that *A. catenella* was present in the water, is error caused by sub-sampling, or an environmental variable not recorded in this study, such as nutrient limitation. Phosphorus limitation in particular is known to lead to an increase in cell-toxin quota in some populations of PSP-producing phytoplankton entering the stationary phase (Anderson *et. al.*, 1990).

The average cell-toxin quota of 39.4 pg STX eq.cell^{-1} is an order of magnitude higher than previously recorded cell-toxin quotas for *A. catenella* found off the west coast of South Africa of 4.8 pg STX eq.cell^{-1} (Ruiz Sebastián *et al.*, 2005) and 1.75 pg STX eq.cell^{-1} (Pitcher *et al.*, 2001). These cell-toxin quotas previously recorded were either from cultured populations, which are expected to have lower cell-toxin quotas (White, 1986; Cembella, 1998) or from a single sample as opposed to the daily samples collected over a 22 day period.

4.2.2 *Alexandrium catenella* toxin profile and toxin biotransformation in the mussel

While the toxin profile for a single strain in a natural population is conserved, those for different strains of *A. catenella* found globally show a great deal of variability (Cembella, 1998). The population of *A. catenella* in this survey showed a relatively conserved toxin profile consisting largely of the more toxic carbamate derivatives (70%), including STX (10%) and NEO (60%). Previously recorded toxin profiles for *A. catenella* found off the west coast of South Africa at Elands Bay in 1999 (Pitcher *et al.*, 2001) and a cultured isolate (Ruiz Sebastián *et al.*, 2005; showed a far greater proportion of the less toxic N-sulfocarbamoyl derivatives (50-60% B1, C1,2) and much lower proportion of NEO in particular. Although the relative proportion of toxins is different, toxin profiles for *A. catenella* off the west coast of South Africa show a very similar suite of toxins: STX; NEO; GTX2,3; C1,2 are common to all records.

A high proportion of N-sulfocarbamoyls is common to most *Alexandrium* populations from Alaska and Pacific coast populations (B1,2 and C1,2) (Cembella, 1998), Chile (B1,2 and C1) (Krock *et al.*, 2007), as well as Japanese populations (C1,2) (Yoshida *et al.*, 2000).

The toxin composition of a vector organism such as the mussel *C. meridionalis* is typically different from the toxin profile of the causative toxigenic phytoplankton. This is a result of the selective retention and elimination of individual toxins as well as metabolic processes within the mussel (Bricelj & Shumway, 1998). The metabolic processes within the mussel that result in a different toxin composition compared to the toxin profile of *A. catenella* include epimerisation, reduction, acidic hydrolysis and enzymatic hydrolysis. As the GTX1,2,3 and C1,2 derivatives have been grouped with α and β epimers together it is difficult to track the characteristic epimerisation of β epimers favoured in PSP-producing phytoplankton to α epimers favoured in contaminated shellfish (Oshima, 1995). GTX1 (an α epimer) is detected in the mussel but not in *A. catenella*, although this could also be due to accumulation of trace amounts too small to measure in the phytoplankton. There is also a significant increase in the proportion of C1,2 derivatives in the mussel compared to *A. catenella*, which could be due to an increase in C1 (the α epimer). The desulfonation and epimerisation of C2 from *A. catenella* to GTX2,3 observed in bivalves (Krock *et al.*, 2007) is not observed here. The significant decrease in the proportion of NEO and increase in the proportion of STX in the the mussel compared to *A. catenella* could be due to the reduction of NEO to STX. The appearance of trace amounts of decarbamoyl derivatives dcSTX and dcGTX2,3 could either be attributed to accumulation of trace amounts from the phytoplankton or enzymatic conversion of the carbamoyl derivatives STX and GTX2,3 (Bricelj & Shumway, 1998).

4.2.3 Uptake and clearance of PSP toxins by the mussel

The net uptake or clearance of toxins measured in the mussel *C. meridionalis* is dependent on many factors, internal and external. Internal factors include feeding rate and selective feeding behaviour of the mussel, metabolic processes, leakage and excretion of toxins, mussel growth and condition.

External factors that influence the rate of uptake and clearance of toxins include the characteristics of the phytoplankton assemblage and environmental conditions (such as temperature) (Bricelj & Shumway, 1998).

Despite the relative low concentration of PSP toxin-producing *A. catenella* cells after 23 March, overall PSP toxin concentrations in the mussel *C. meridionalis* did not show any noticeable decrease until a week later after 30 March. Possible reasons for this delay in net clearance of toxins includes the capacity for the mussels to retain toxins without adverse effect, growth and condition of the mussels, low abundance of non-toxic food sources, and environmental conditions. Other shellfish such as the scallop *Pactinopen yessoensis* is capable of retaining PSP toxicity for months following the decline of the *A. catenella* population that caused the toxicity (Sekiguchi *et al.*, 2001).

As for other shellfish, the mussel *C. meridionalis* is relatively resistant to the effect of toxins, which would allow it to retain a high concentration of toxin. The survey period was too short to encompass any significant change in weight of the mussels due to growth, which would have had a diluting effect on toxin concentration (Bricelj & Shumway, 1998). The harvesting and transference of the mussels from their intertidal habitat to the mooring could have contributed to a drop in the condition of the mussels which would further slow the growth of the mussels. During subsequent monthly harvesting of mussels the shells displayed deformities indicating a loss of condition and slowed growth. Although wide meshed bags were used to ensure water flow mussels tended to aggregate and also became increasingly fouled and encrusted over time, decreasing the flow of water to the mussels located on the inside of the aggregate. This could have reduced the availability of subsequent alternative food sources and slowed the growth of the mussels and subsequent dilution of toxin concentrations further. The decrease in water temperature during the second upwelling event centred around 27 March may also have contributed to increased retention time for toxins, as it would have slowed down physiological processes in the mussel, including detoxification.

A possible reason for the increased rate of clearance of PSP toxins by the mussels after 30 March is the introduction of an alternative food source, the high cell concentrations of diatoms such as *Pseudo-nitzschia*, which could lead to a lower proportion of *A. catenella* being ingested. This co-coincided with an increase in water temperature that would encourage physiological processes in the mussel (including detoxification). Total phytoplankton biomass (indicated by chlorophyll-*a* values) rapidly declined during the second upwelling period centred around 27 March and only peaked again between 28 - 30 March, just before the onset of clearance of PSP toxins. Therefore although *A. catenella* cell concentrations were relatively low following the first upwelling event centred around 21 March (a few thousand cells.L^{-1}) they may have been sufficient to maintain toxin concentrations in the mussel. Low water temperature and the absence of alternative food sources might also have contributed to preventing the elimination of toxins in the mussel.

4.2.3.1 Comparison of methods used to quantify PSP toxins in the mussel

Of the methods used to detect PSP toxins in the mussel hydrophilic interaction liquid chromatography linked with tandem mass spectrometry (HILIC-MS/MS), the enzyme-linked immunosorbent assay (ELISA) and the receptor binding assay (RBA) showed the greatest agreement, while all methods showed a relatively poor agreement with the mouse bioassay (MBA). Compared to ELISA and RBA, HILIC-MS/MS tended to give slightly higher results (with a mean ratio > 1), this has been observed before (Costa *et al.*, 2009). In the case of the ELISA it is attributed to low cross reactivity with the derivatives of STX which would lead to underestimation of total toxicity. The ELISA test for PSP toxins recognizes mostly saxitoxin (STX) (showing 100% cross reactivity) and other PSP toxins to a much lesser degree (29% cross reactivity and below). As ~25% of the mussel toxin profile is made up of other PSP toxins this can lead to an underestimated result. The RBA quantifies total toxicity using toxic effect, and therefore the ~25% of less toxic PSP toxins would also lead to a slight underestimation compared to a chemical analytical method such as HILIC-MS/MS. The tendency of the MBA to underestimate the results has also been noted before (Costa *et al.*, 2009), especially above toxin concentrations of 100 µg STX eq.100 g^{-1} mussel where it has been observed to differ from 20 - 50% (Holtrop *et al.*, 2006).

4.3 Diarrhetic shellfish poisoning toxins

4.3.1 Cell-toxin quota for *Dinophysis* spp.

The relationship between *Dinophysis* spp. cell concentration and DSP toxin concentration in the particulate fraction is positively linear, and although it is statistically significant there is a great deal of variability. Cell-toxin quotas for *Dinophysis* spp. often show a great deal of seasonal variation, even within one species from the same area (Wright & Cembella, 1998). Some recent studies show that toxicity can be an ephemeral trait, and populations of *D. acuminata* have shown high variability in cell-toxin quota even within the same day (Marcaillou e*t al.*, 2005). The difficulty in maintaining *Dinophysis* in laboratory cultures has meant that the mechanisms of DSP toxin production are not well understood. Recent work by Hackett *et al.* (2009) demonstrates that previously non-toxigenic *D. acuminata* cultures can be induced to produce DSP toxins. Their study showed that variation in toxin production may be a product of different environmental conditions (that either induce or repress toxin production), genetic differences in the *Dinophysis* spp. that ultimately modify toxin production, or differences in prey species which are the plastid source of *Dinophysis*, with some plastids showing more effective toxin production or sequestration. Another reason for variability in cell-toxin quota is error due to sub-sampling.

Typically *Dinophysis* spp. form a relatively small percentage of the total phytoplankton assemblage off the west coast of South Africa, however they have been known to reach cell concentrations in the order of 10^6cells.L^{-1} (Pitcher & Calder, 2000). The results recorded here of a maximum of 0.01 µg OA.L^{-1} corresponding to 8.3x10^4cells.L^{-1} fall within the previously recorded ranges for

this region where a maximum of 0.4 µg OA.L^{-1} corresponded to 4.2x10^4cells.L^{-1} (Fawcett *et al.*, 2007).

Dinophysis spp. found off the west coast of South Africa have been reported as moderately toxic (Trainer *et al.*, in press b; Pitcher & Calder, 2000). The cell-toxin quota recorded in this study of 0.07 pg OA.$cell^{-1}$ indicate a low toxicity put into context with global cell-toxin quotas for *Dinophysis* spp., which range from a few pg OA.$cell^{-1}$ to hundreds of pg OA.$cell^{-1}$ ((Moroño e*t al.*, 2003; Marcaillou *et al.*, 2005).

4.3.2 *Dinophysis* spp. toxin profile and toxin biotransformation in the mussel

Dinophysis spp. appear to produce mainly okadaic acid (OA) and the two dinophysistoxin derivatives, DTX1,2, although this might be an artefact of the methods traditionally used to detect DSP toxins which might have underestimated pectenotoxins (Wright & Cembella, 1998; MacKenzie *et al.*, 2005). Off the west coast of South Africa OA is the primary toxin produced by toxigenic *Dinophysis* spp., and although several potentially toxigenic species have been recorded, DSP events are mostly attributed to *D. acuminata* and *D. fortii* (Trainer *et al.*, in press b). Lower concentrations of pectenotoxins (particularly PTX2) have also been recorded, and correspond with the presence of *D. acuminata* and *D. fortii* (Fawcett *et al.*, 2007). The toxin profile for *Dinophysis* spp. recorded for this survey is consistent with previous records, with OA forming the bulk of the toxin produced (~95%), with the remaining 5% split between DTX1 (~1%) and PTX2 (~4%).

OA and dinophysistoxins are not always the dominant toxins in toxin profiles for *Dinophysis* spp., in New Zealand *D. fortii*, *D. acuta* and *D. norvegicus* have been associated with toxin profiles that have a much higher proportion of PTX (MacKenzie *et al.*, 2005).

After ingestion of toxigenic phytoplankton by the mussel *C. meridionalis* the composition of toxins typically changes. The toxic okadaic acid group compounds (OA, DTX1,2) can all be acylated by metabolic processes in shellfish, and these acylated forms (collectively known as DTX3) have not been recorded in the toxigenic phytoplankton (Moroño *et al.*, 2003). The acylated forms (DTX3) can be hydrolysed in the gastrointestinal tract to OA, DTX1 or DTX2. Some strains of *D. acuminata* can store OA in a non-toxic diol-ester form which is rapidly converted to OA by shellfish (Hackett *et al.*, 2009). Slower rates of acylation of OA to its conjugated forms (including diol-esters and DTX4-type toxins) are expected to occur in shellfish as well (Moroño *et al.*, 2003). The mussels in this survey showed a significant increase in the proportion of DTX1 relative to OA. This could indicate a faster elimination of OA compared to DTX1 or transformation of OA into different forms. No acylated or conjugated forms of OA or DTX1 were detected in the mussels that would indicate transformation of these compounds, therefore it would appear that mussels eliminate the more toxic OA more efficiently than DTX1.

Of the pectenotoxins only PTX2 was detected in the phytoplankton, whereas both PTX2 and PTX2sa were recorded in the mussel, with a relatively higher proportion of PTX2sa. With pecteno-

toxins the enzymatic conversion of PTX2 to PTX2sa has been shown to occur in shellfish (Quilliam, 2003a).

4.3.3 Uptake and clearance of DSP toxins by the mussel

The mussel *C. meridionalis* showed a steady net uptake of DSP toxins throughout the survey period from far below the regulatory limit of 16 µg OA eq.100g^{-1} of mussel to well above, reaching a maximum of 43 µg OA eq.100g^{-1} of mussel. A rapid increase in the DSP toxin concentration in the mussels was recorded after 3 April despite the relatively low numbers of toxigenic *Dinophysis* spp. Toxigenic *Dinophysis* spp. showed cell concentrations two orders of magnitude below that of diatoms of the *Pseudo-nitzschia* spp. occurring at the same time, and showed a relatively low cell-toxin quota. Globally, outbreaks of DSP are known to occur even in mixed phytoplankton assemblages where relatively low numbers of toxigenic *Dinophysis* spp., <10^{3}cells.L^{-1}, are recorded (Belin, 1993). Off the west coast of South Africa several DSP events have been recorded, with cell concentrations of *Dinophysis* spp. often >10^3 cells.L^{-1}, but also forming a relatively small percentage of the total phytoplankton assemblage (Pitcher *et al.*, 1993).

Despite two peaks in *Dinophysis* spp. cell concentration of 8.3x10^4 cells.L^{-1} on 25 March and 4.7x10^4 cells.L^{-1} on 29 March an increase in the rate of toxin uptake was only observed after 3 April, exceeding the regulatory limit of 16 µg OA eq.100g^{-1} mussel by the end of the survey period (11 April). Unlike the other toxigenic dinoflagellate present during the survey, *A. catenella*, which stayed at relatively low cell concentrations after initially very high cell concentrations, *Dinophysis* spp. showed more persistent cell concentrations throughout the survey period. Mussels are known to have a higher rate of toxin uptake than of toxin clearance (Bricelj & Shumway, 1998) and the persistence of even relatively low cell concentrations of *Dinophysis* spp. of low toxicity could result in a net increase in DSP toxin concentration in the mussel. The increased rate of DSP toxin uptake by the mussels after 3 April could be attributed to the decline of the much higher cell concentrations of other phytoplankton, particularly *Pseudo-nitzschia* spp., that had previously been present together with the *Dinophysis* spp. and could have acted to decrease the relative proportion of *Dinophysis* filtered and ingested by the mussel. Another possible reason for the increasing DSP toxin concentrations in the mussels despite the relatively low cell concentrations and cell-toxin quota of *Dinophysis* spp. is the conversion of a non-toxic OA derivative produced by the toxigenic phytoplankton into OA by the mussel. The conversion of non-toxic derivatives to toxic OA by mussels has been recorded by Hackett *et al.* (2009).

As for PSP toxins, the environmental conditions might have contributed to the slower initial uptake of toxins by the mussel, as uptake of DSP toxins increased rapidly after the water column had warmed and stratified in the relaxation period after the second upwelling event. The lower temperature during the two upwelling events would have slowed metabolic processes in the mussel, such as the conversion of non-toxic derivatives to toxic OA.

4.3.3.1 Comparison of methods used to quantify DSP toxins in the mussel

The two methods used to detect DSP toxins in the black mussel, liquid chromatography linked with tandem mass spectrometry (LC-MS/MS) and the enzyme-linked immunosorbent assay (ELISA), showed good agreement throughout the survey period. ELISA has a decreased cross-reactivity with the DTX derivatives of okadaic acid, and as these are present in a relatively low proportion in the mussel (20%) it would be expected to lead to a slight underestimation of the concentration of total DSP toxins but this was not observed in this study.

4.4 Amnesic shellfish poisoning toxins

4.4.1 Cell-toxin quota for *Pseudo-nitzschia* spp.

The cell-toxin quota of *Pseudo-nitzschia* spp. recorded has a measure of uncertainty as there was no identification further than genus level and therefore no way of determining what fraction of the total concentration of cells was responsible for toxin production. Intracellular domoic acid in natural populations is highly variable, attributed to the fact that toxin production is most likely to be a stress response to limited nutrients and elevated pH and therefore environmentally dependent (Trainer *et al.,* in press).

Cell concentrations of 0 - 1.2x10^6 cells.L^{-1} for *Pseudo-nitzschia* spp. fall within the range detected in the southern Benguela previously of 0 - 1.6x10^7 cells.L^{-1} (Fawcett *et al.*, 2007). The particulate domoic acid concentration of 0 - 0.45 µg DA.L^{-1} is lower than previous records from 0.1 - 3 µg DA.L^{-1} (Fawcett *et al.*, 2007). These are similar to toxigenic *Pseudo-nitzschia* cell concentrations and particulate DA concentrations found globally, for example cell concentrations and particulate DA concentrations recorded for *P. pseudodelicatissima* off the Washington coast (0.2 & 0.6x10^6 cells.L^{-1} , corresponding to 0.2 & 2.7 µg DA.L^{-1} recorded in 1997 and 1998 respectively) (Trainer *et al.*, 2002).

Presuming that all cells counted were toxigenic, 0.19 pg DA.$cell^{-1}$ indicates a relatively low cell-toxin quota in a global context, an order of magnitude lower than that of *P. multiseries* (6 pg.$cell^{-1}$) and 2 orders of magnitude lower than that of *P. australis* (78 pg.$cell^{-1}$) found near California coastal upwelling cells, recorded in 1998 (Trainer *et al.*, 2000).

4.4.2 The absence of ASP toxins in the mussel

There were relatively high densities of *Pseudo-nitzschia* spp. present throughout most of the survey period, the maximum cell concentration of 1.2x10^6 cells.L^{-1} exceeding that usually associated with ASP events (Bates *et al.*, 1998). The ASP toxin domoic acid was also detected in the particulate fraction, however no domoic acid (DA) was detected in the mussel *C. meridionalis*. A possible explanation for this is the low cell-toxin quota for *Pseudo-nitzschia* spp., which might

have been too low to lead to detectable toxin accumulation. However similar cell-toxin quotas and cell concentrations of *Pseudo-nitzschia* spp. in California coastal waters as those observed off the west coast of South Africa have resulted in ASP events. Therefore low cell-toxin quotas are not likely to be the only reason for the continued absence of records of ASP events off the west coast of South Africa.

In regions where ASP outbreaks occur such as the Californian coastal waters, mussels are not always the primary vectors of ASP toxins, or do not accumulate DA during blooms of toxigenic *Pseudo-nitzschia* spp. at all (Scholin *et. al.*, 2000; Ferdin *et al.*, 2002). Even though DA is present in other vector species such as planktivorous anchovy *Engraulis mordax* in concentrations that are high enough to result in illness and death in the marine fauna that feed on the contaminated anchovies, it is not present in detectable concentrations in the local mussel populations. DA also been detected in organisms such as the sand crab *Emerita analoga* (Stimpson) while the mussel *Mytilus californianus* did not have any detectable concentrations. This could indicate a size-selective feeding preference by the mussels, Zebra mussels (*Dreissena polymorpha*) have been observed to reject larger planktonic phytoplankton such as diatoms while preferentially feeding on smaller plankton such as the cyanobacterium *Microcystis* (Baker *et al*., 1998; Vanderploeg *et al*., 2009).

Yet another possible reason for not detecting DA in the mussel samples is the possibility of loss of toxicity due to photodegradation, which is known to occur to DA in seawater samples (Bates *et al.*, 2003; Bouillon *et al.*, 2006) and can therefore be expected to occur in mussel samples as well. While the filters were protected from light using foil the mussel samples were stored in clear containers and exposed to light. In future it is recommended that for samples potentially contaminated with DA care should be taken to prevent exposure to light.

Chapter 5

Conclusions

Understanding the relationship between cell concentrations, the toxicity of toxigenic phytoplankton, and the resultant shellfish toxicity is important in the design of effective programmes for the monitoring of the safety of shellfish for consumption. During this 22-day survey three groups of toxigenic phytoplankton were present in the water: *A. catenella*, producing paralytic shellfish poisoning (PSP) toxins; *Dinophysis* spp., producing diarrhetic shellfish poisoning (DSP) toxins; and *Pseudo-nitzschia* spp, producing amnesic shellfish poisoning (ASP) toxins. Of these groups of toxins only the PSP and DSP toxins were detected in the mussel *C. meridionalis*.

The use of light microscopy to identify and enumerate different toxigenic species was adequate for the identification of *A. catenella* and the *Dinophysis* spp., however *Pseudo-nitzschia* spp. are more cryptic and require the use of electron microscopy or molecular techniques in order to identify different species. In the routine monitoring of toxigenic phytoplankton the identification of *Pseudo-nitzschia* species therefore poses the greatest challenge, as it is likely that not all of the species present are toxigenic. As there has been no record of ASP toxicity in shellfish off the west coast of South Africa despite the presence of ASP producing phytoplankton in the water column further investigation is required. An important first step is to establish which *Pseudo-nitzschia* spp. found off the west coast of South Africa are toxigenic. Mussels are most often used as indicators of the presence of toxigenic phytoplankton as they accumulate and eliminate toxins relatively quickly, however the feeding behaviour of mussels could influence which toxigenic phytoplankton are ingested. The possibility that there is an indicator species for ASP toxins that is more likely to accumulate domoic acid when toxigenic *Pseudo-nitzschia* are found in the water should also be considered.

The daily records of field data presented in this study provide the necessary resolution to understand the dynamic relationship between toxicity of phytoplankton and toxin concentrations and biotransformation of toxins in a vector species such as the mussel *C. meridionalis*.

The *in situ* uptake and depuration of toxins by the mussel *C. meridionalis* is complex, dependent not only on the cell concentrations and toxicity of toxigenic phytoplankton, but also on feeding behaviour and environmental conditions that effect metabolic processes (including transformation or elimination of toxins). The expected delay between changes in concentration of toxigenic phyto-

plankton and resultant uptake and depuration was observed, especially for PSP toxins. PSP toxins were depurated by the mussel a week after the decline of the highly toxic *A. catenella* bloom from $6.1x10^5$ cells.L^{-1} to a few 1 000 cells.L^{-1}. The elimination of PSP toxins coincides both with an increase in alternative food sources (high cell concentrations of *Pseudo-nitzschia* spp.) and an increase in water temperature. The absence of ASP toxins that were detected in the *Pseudo-nitzschia* spp. indicates that it is unlikely that the mussels ingested these diatoms, which would have decreased the relative proportion of PSP toxin-producing *A. catenella* ingested. The high cell concentrations of *Pseudo-nitzschia* spp. is therefore an unlikely reason for the onset of PSP toxin elimination. The depuration of PSP toxins by the mussel depends not only on the decline of the toxigenic phytoplankton *A. catenella* but also on environmental conditions, the increase in water temperature later in the survey period presumably leading to the increased elimination of PSP toxins due to an increase in metabolic rates in the mussel. The relative importance of water temperature and toxigenic cell concentration is unclear and would need further study in a laboratory where the variables could be independently tested.

The rate of uptake of DSP toxins by the mussel was steady despite low toxicity displayed by *Dinophysis* spp. and fluctuations in the cell concentrations of *Dinophysis* spp., which peaked twice at $8.2x10^4$ cells.L^{-1} and $4.7x10^4$ cells.L^{-1}, seldom falling below 1 000 cells.L^{-1}. An increase in the rate of DSP toxin uptake by the mussel occurred a few days after the second peak. Similarly to PSP toxins, DSP toxin uptake is also dependent not only on the presence of toxigenic *Dinophysis* spp. but also on environmental conditions. The increase in water temperature presumably resulting in an increased transformation of non-toxic diol esters to the toxic OA form as metabolic rates in the mussel increase. This would be supported by evidence for the presence of these diol ester forms in the toxigenic *Dinophysis* spp. off the west coast of South Africa, which would require identification using a highly analytical chemical method such as liquid chromatography linked to tandem mass spectrometry.

Despite relatively large cell concentrations of toxigenic *Pseudo-nitzschia* spp. (up to $1.2x10^6$ cells.L^{-1}) no ASP toxins were detected in the mussel. This indicates that either the methods used to detect ASP in shellfish are inadequate, or that the mussel *C. meridionalis* selectively excludes the toxigenic diatoms while filter-feeding, co-incidentally preventing ingestion of ASP toxins. This could be confirmed using a selective feeding experiment.

Cell concentrations and toxicity of toxigenic phytoplankton cannot be used in isolation to predict the rate of toxin uptake or depuration for vector organisms such as the mussel *C. meridionalis*. The rapid changes in phytoplankton assemblage reported here, and the varied responses of the same mussel species to different toxigenic phytoplankton, demonstrate the difficulty in using cell concentrations in determining "action limits" in the management of shellfish monitoring programmes.

The toxin profile for *A. catenella* showed an unusually high proportion of NEO compared to previous records off the west coast of South Africa and globally, which typically have higher proportions of C1,2. Toxin profiles have been considered as useful markers for phytoplankton populations from different geographical areas, which could show paths of dispersal due to natural phenomena or human intervention (such as dispersal via ballast water in ships). This study supports previous

observations that the PSP toxin profile is conserved in a single population of *A. catenella*, however the difference in the profile reported here compared to those previously reported for this region could indicate the introduction of *A. catenella* from different regions has occurred, and this population may not be representative of the typical toxin profile for *A. catenella* off the west coast of South Africa. *Dinophysis* spp. and *Pseudo-nitzschia* spp. showed more typical toxin profiles compared to previous records off the west coast of South Africa.

Biotransformation of toxins from *A. catenella* by the mussel *C. meridionalis* shows the expected reduction of NEO to STX. The desulfonation and epimerisation of C2 to GTX was not observed, perhaps due to the low proportion of C1,2 derivatives. The biotransformation of toxins from the *Dinophysis* spp. by the mussel also showed the typical reduction in OA relative to DTX1, as well as the metabolism of PTX2 to PTX2sa.

Comparisons of different methods used to quantify shellfish toxin concentrations also provide an indication of which methods are most appropriate. Several different methods were used to quantify shellfish toxin concentrations in this study, including chemical analytical methods, *in vitro* and *in vivo* assays. All these methods showed similar trends, with particularly good agreement for the toxin concentrations for the less structurally diverse DSP toxins. The chemical analytical method liquid chromatography linked with mass spectrometry (LC-MS/MS) or the *in vitro* enzyme linked immunosorbent assay (ELISA) showed the most similar results, the DSP toxin concentrations in this study ranged around the regulatory limit of 16 µg OA eq.100g^{-1} (3.8 - 43.8 µg OA eq.100g^{-1} mussel). For the more structurally diverse PSP toxins there was a higher variability, especially for the *in vivo* mouse bioassay (MBA), which underestimated PSP toxin concentrations compared to the other methods. Methods used to quantify PSP toxins at very high concentrations (489 - 1.2x10^4 µg. STX eq.100g^{-1} mussel) far above the regulatory limit of 80 µg STX eq.100g^{-1} mussel. that agreed well in this study were the chemical analytical method LC-MS/MS, and the two *in vitro* assays, the receptor binding assay (RBA) and the ELISA.

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Appendices

Appendix A

Table 1: Comparison of best-fit models using a non-linear regression analysis, one-phase exponential decay, of the depuration of individual PSP toxins by the mussel *Choromytilus meridionalis*, created using GraphPad Prism v.5. The null hypothesis was no change in concentration of PSP toxins (where rate of change would equal 0), and the constraints were added that the rate could not be negative (i.e. toxins were lost not gained) and that at 0 concentration the rate would be 0. The goodness of fit for the model was measured using r^2 (values approaching 1 indicate a good fit). The normality of residuals (shown as an insert in Fig. 3.8 on page 47) were measured using D'Agostino and Pearson's test, residuals following a normal distribution would have a P value of greater than 0.5

Comparison of Fits	**STX**	**dcSTX**	**NEO**	**GTX1**	**GTX2,3**	**dcGTX2,3**	**B1**	**C1,2**
P value	<0.0001		0.0003	0.2314	<0.0001	0.5901	0.0003	0.0002
Preferred model	AH	NH	AH	AH	AH	NH	AH	AH
Null hypothesis	K=0							
Alternative hypothesis	K>0							
Best-fit values								
Y0	12102	4.914	20536	127.3	256.4	86.19	469.4	13365
K	0.1055	$2.011E^{-13}$	0.202	0.05934	0.06184	0.009248	0.05587	0.08781
Goodness of Fit								
R^2	0.9292	$-1.163E^{-5}$	0.75	0.1397	0.8136	0.03004	0.7499	0.7742
Normality of Residuals								
D'Agostino & Pearson	4.735	3.722	1.243	2.567	6.224	0.7424	1.773	0.2943
Constraints								
Plateau	Plateau = 0							
K	K≥0							
Degrees of Freedom								
DF	10							

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